THE FACTORS OF COAGULATION IN THE EXPERIMENTAL APLASTIC ANEMIA OF BENZOL POISONING, WITH SPECIAL REFERENCE TO THE ORIGIN OF PROTHROMBIN.*

By S. H. HURWITZ, M.D., and C. K. DRINKER, M.D.

(From the Laboratory of Surgical Research of the Harvard Medical School, and the Medical Clinic of the Peter Bent Brigham Hospital, Boston.)

INTRODUCTION.

In the course of a study (1) on the factors of coagulation in a case of aplastic anemia we were impressed with the analogy existing between the symptom-complex in this disease and that observed by Selling (2, 3) in three cases of benzol poisoning. The chief clinical features presented by Selling's three patients were: first, purpura hemorrhagica, with typical skin lesions, bleeding from the mucous membranes, and retinal hemorrhages; second, a blood picture characterized by a leucopenia of a marked grade, a striking reduction in the blood platelets, and anemia. Pathologically these patients showed well marked aplasia of the bone marrow. A study of his three patients induced Selling to try to produce in animals aplastic anemia with benzol. This he accomplished successfully in rabbits and showed that benzol is a powerful leucotoxic agent, acting chiefly upon the bone marrow, although producing changes of some degree also in other hematopoietic organs.

Workers who have studied the blood in benzol poisoning have concerned themselves thus far chiefly with the formed elements. Selling (3) found both a reduction in the red corpuscles and a striking diminution in the leucocytes, with an almost total disappearance of these elements in the blood of fatally poisoned animals. In addition, Duke (4) found a marked drop in the platelet count. According to Duke, a sufficiently low reduction in blood plates will lead to the prolonged bleeding time noted in benzol intoxication. Duke's studies included also occasional determinations of the blood fibrinogen; but we have been unable to find any complete and systematic studies of the factors of coagulation in experimental conditions associated with aplasia of the bone marrow.

The present series of experiments was undertaken with the hope of learning something about the factors of coagulation in benzol poisoning, but as the work progressed it seemed to give additional information concerning the relation of the bone marrow to the origin of the factors of coagulation studied by us; namely, prothrombin, antithrombin, and fibrinogen.

* Received for publication, February 8, 1915.

Our observations have shown that the clinical symptoms of an animal poisoned with benzol, as far as the hemorrhagic features are concerned, give only slight indication of the profound changes which this myeloid tissue poison produces in the blood of such animals. The latter do not, as a rule, exhibit the usual hemophilic tendency with which we are familiar,—delayed bleeding time, purpura, bleeding from the gums and mucous membranes, etc. Very occasionally one meets with prolonged bleeding from cuts and ear pricks, which, according to the recent observation of Duke (5), occurs only when the plates are reduced to a dangerously low level, 5,000 to 15,000 per cubic millimeter. With the exception of a few instances in which the blood, at autopsy, was found to remain fluid for a long time, no striking departure from normal clotting was noted. A study of the blood showed, however, that striking changes had resulted both in the formed elements and in the prothrombin content, although such changes were not of a degree sufficiently marked to produce the clinical picture of hemorrhagic disease.

As far as the effect of benzol upon the formed elements is concerned, our experiments confirm the work of Selling and of Duke. Following the administration of benzol, there is a rapid disappearance of the white cells from the peripheral circulation. We have observed also an initial rise in some instances (experiments B, F, G, and H). The red blood corpuscles are much less affected than the white. In a few experiments (B, E, and G) the count remained unaltered, whereas in the majority of observations the red cell count was reduced by 50 per cent. or more.

The platelets showed usually the same general reduction in number, though to a less extent. We were impressed with the observation that the blood plates may remain at a high level at a time when the white cells have almost entirely disappeared from the circulation. In only one instance were we able to reduce the number of platelets so low as to reproduce the symptoms of hemorrhagic disease. Such experiments suggest the possibility that the megakaryocytes of the bone marrow either regenerate very rapidly or else are more resistant to the toxic action of benzol than are the forerunners of the polymorphonuclear leucocytes and erythrocytes.[1] This view,

[1] According to Selling's work (*loc. cit.* (3), p. 53), the polymorphonuclear

if correct, would help to explain the difficulty encountered in reproducing by benzol injections the features to which attention has been called.

Besides the formed elements, we have followed the factors of coagulation in the majority of our animals. These studies showed that benzol exerts an important influence in reducing the circulating prothrombin and that this substance is dependent for its production, in part at least, upon bone marrow activity. Two of the other factors of blood clotting, antithrombin and fibrinogen, were also followed in the majority of the experiments. These were found to fluctuate little from the normal.

Our results are in harmony with the recent proof that blood plates contain prothrombin. They give additional evidence in support of the view that fluctuations in the amount of prothrombin may be produced by substances which affect the number of platelets, and that any toxin which produces a reduction in their number will simultaneously cause a diminution in the available prothrombin of the circulating blood. Our knowledge, moreover, of the origin of blood platelets from the megakaryocytes (6) of the bone marrow emphasizes the importance of this tissue in maintaining the normal prothrombin equilibrium of the blood. Although the marrow plays this important part in the elaboration of prothrombin, no definite parallelism could be established between the extent of bone marrow injury, the number of blood plates, and the relative amounts of prothrombin. This would suggest that it is not possible to create a dangerous prothrombin deficiency by a reduction in the number of blood platelets alone, and that some other tissue or tissues play some part in prothrombin formation.

It will be seen, too, that the marrow in no way participates in the production of antithrombin and fibrinogen, for we did not observe any changes in these substances in the blood of animals with aplastic marrow.

METHODS.

Experimental Benzol Poisoning.—The technique employed by us for producing aplasia of the bone marrow was the same as that used

amphophils in the marrow are more resistant than large lymphocytes, myelocytes, erythroblasts, and giant-cells.

by Selling. Because of the knowledge of the pathological lesions produced by benzol in the rabbit, gained from the fundamental observations of this worker, these animals were chosen for our experiments.

Injections were made subcutaneously with chemically pure benzol. The average dose injected was two cubic centimeters of benzol daily per kilogram of body weight, although smaller doses were used when the animals began to show signs of marked intoxication. Sudden death may follow the injection of too large doses. The acute symptoms accompanying such sudden deaths are referable chiefly to the central nervous system, sufficient time not having been allowed for the development of the myelotoxic effect of this poison. It is essential, therefore, to proceed slowly with smaller doses in order to produce the chronic type of poisoning which results in the typical changes in the hematopoietic organs.

We have found certain animals quite resistant to the poison. In some instances, many injections were necessary in order to produce the desired effect. Such resistance, however, is not the usual rule. Most animals succumb after ten doses, and at autopsy show fairly well marked aplasia of the marrow. It was our original plan to bring about an aplasia of the bone marrow, after which regeneration was to be permitted to occur, and to follow the fluctuations of the prothrombin as the animals were returning to a normal state. It was difficult, however, to obtain such an ascending series because repeated injections were necessary to produce demonstrable changes in the prothrombin content of the blood and these usually resulted fatally.

Variations in the white cell count and in the number of blood platelets give a fair index of the condition of the bone marrow, the counts dropping as the aplasia becomes marked. Selling[2] has pointed out that when the number of white cells has reached as low a level as 200 to 800 cells per cubic millimeter, any additional injections usually prove fatal. This has been our experience also.

Thus far we have been unable to reduce the blood platelets to a dangerously low level. In only three experiments did the number of platelets fall below 100,000, and in only one instance to 30,000. This observation may be of interest in connection with a point to

[2] Selling, *loc. cit.* (3), p. 11.

which attention will be directed later, that it is also impossible by this method to diminish sufficiently the prothrombin of the circulating blood so as to render these animals hemophilic.

Obtaining Blood.—The problem of obtaining specimens of blood from rabbits with the precautions which are necessary for this work was solved by resorting to cardiocentesis with a graduated Luer syringe. Before introducing the needle the interior of the syringe was coated with a thin layer of a mixture of white petrolatum 25 parts and ether 300 parts, and the needle was filled with salt solution. With a little practice it is possible to obtain blood in this way after one attempt. This precaution is important, since repeated thrusts of the needle into the heart may traumatize its muscle, setting free neutralizing tissue juices, and frequently producing a hemopericardium which may embarrass the heart's action and cause sudden death. By the exercise of skill it is possible to repeat cardiocentesis many times without endangering the life of the animal.

For our purposes ten cubic centimeters of blood sufficed. This was immediately emptied into a centrifuge tube containing two cubic centimeters of a 1 per cent. solution of sodium oxalate (made up in 0.9 per cent. solution of sodium chloride). After being thoroughly mixed the specimen was centrifugalized at a constant speed (a definite number of revolutions on our centrifuge) for ten minutes. The plasma, which in rabbits is usually watery or opalescent, due to a slight lipemia, was pipetted off and examined for the various factors of coagulation, according to the methods of Howell (7, 8).

Enumeration of Formed Elements.—Of all the methods proposed for the enumeration of blood platelets, that of Wright and Kinnicutt (9) appears to have given the most reliable results in the hands of recent workers. As used by Duke (5) this method has given constant results and was the one we used.

Quantitation of Prothrombin, Antithrombin, and Fibrinogen.—In the writings of Howell and his coworkers will be found a comprehensive description of the methods introduced by him for determining the amount of prothrombin and antithrombin in blood. In a recent paper the authors have already commented upon the various methods and their usefulness. For purposes of clearness, however, we wish to mention them briefly at this time, particularly with refer-

ence to certain precautions to be followed in carrying out the prothrombin test.

Prothrombin.—As yet no good method is available for the isolation and absolute quantitation of prothrombin in blood plasma. An idea of the relative amount or relative strength of the prothrombin in the blood may be obtained, however, by a simple method devised by Howell.[3] This depends upon the observation that the act of oxalating, that is, the decalcifying of the solution, intensifies the activation of prothrombin to thrombin by a subsequent calcification. This results apparently in a greater production of available thrombin for interaction with the fibrinogen of the plasma.

In practice the reaction is carried out as follows: To a series of tubes containing a constant amount of oxalated plasma is added dilute calcium chloride in varying amounts. Coagulation will result and the time of coagulation, as measured by the invertibility of the clot, will be shortest in the tube containing the optimum amount of calcium. Tested by this method, the clotting time of normal rabbit plasma averages about ten minutes.

To insure uniform results, it is necessary to take two precautions: first, the employment of an equal quantity of each reagent; second, centrifugalization of the plasma at a constant speed and for a constant number of minutes in all comparable observations. That the speed of this prothrombin reaction can be influenced by centrifugalization was shown by Lee and Vincent (10) and again emphasized by Howell in a recent publication (11). This was shown to depend upon variations in the thromboplastin content of plasma centrifugalized for different periods of time. Failure to observe these two points may lead to erroneous results in experiments where no wide differences exist between the quantity of prothrombin present in normal and in pathological blood.

Antithrombin.—The method of demonstrating the anticoagulating action of normal and pathological plasmas has been so clearly described in recent papers that only brief mention of the technique need be considered here. The test plasma is heated slowly to 60° C. and then centrifugalized to remove the fibrinogen and prothrombin. A drop of this plasma containing the antithrombin is then added to

[3] Howell, *loc. cit.* (7), p. 78.

known amounts of thrombin solution. After a short period of incubation, fibrinogen solution is added. The addition of the antithrombin delays or inhibits completely the action of thrombin on fibrinogen. In tests in which the thrombin is nearly neutralized by the antithrombin, it is very difficult to ascertain when clotting has occurred, since under such circumstances the clot forms in several stages. For purposes of uniformity we have adopted as the end-point of this reaction the first appearance of a delicate or filmy clot. In clinical tests the relative amount of antithrombin in the oxalated plasma of the patient is compared always with a similar specimen from a normal person; in our animal experiments the initial observation made upon the healthy animal always under exactly the same conditions, and with the same thrombin and fibrinogen solutions where possible, served as the control. Reference to one of the protocols given below will help to make clear the method of making the comparisons.

Fibrinogen.—The heat coagulation method of determining fibrinogen has also been described many times in recent communications. For details of this method reference should be made to papers by Whipple and Hurwitz (12) and by Whipple (13).

EXPERIMENTAL DATA.

With the exception of one or two experiments, to which attention will be directed later, the observations may be divided into two main groups: first, those in which the animals showed great susceptibility to the poison, as indicated clinically by a great drop in the formed elements, and pathologically by an aplasia of the bone marrow of varying degree; second, those in which less striking reduction in the formed elements, particularly in the blood platelets, could be produced, and in which autopsy revealed either a less well marked aplasia or in a great many instances a marrow in which the regenerative process was keeping pace with the destructive changes. In the first group a diminution in the circulating prothrombin was demonstrable; whereas in the second group little or no change from the normal was observed.

In all, about fifteen complete experiments were performed. Of these three following two, one from each group, will be summarized,

since they are fairly complete and quite representative of all the other experiments.

TABLE I.

Rabbit P.

Date.	Weight in gm.	Dose of benzol in c.c.	Formed elements.			Factors of coagulation.				
			Platelets.	W. B. C.	R. B. C.	Prothrombin.		Antithrombin.[4]		Fibrinogen in gm. per 100 c.c.
						$CaCl_2$	Min.	Thrombin.	Min.	
Nov. 11	1,400	2	920,000	7,800	5,200,000	1	11	3	15	0.719
						2	15	4	5	
						3	15	5	5	
						4	15			
Nov. 12–15		6								
Nov. 15	1,350	2	480,000	3,200	4,700,000	1	40	3	No clot in 60	0.656
						2	31	4	6	
						3	30	5	8	
						4	38			
Nov. 16–18		4								
Nov. 19	1,175	1	185,000	1,900	3,710,000	1	60	3	23	0.684
						2	60	4	7	
						3	60	5	5	
						4	60			
Nov. 20–24		8								
Nov. 25	1,100		128,000	500	1,810,000	1	50	3	5	0.638
						2	55	4	12	
						3	65	5	5	
						4	95			

PROTOCOL.

Rabbit P.—(Table I.) Nov. 11. Young brown male; weight 1,400 gm. Red blood count 5,200,000; white blood count 7,800; platelets 920,000; fibrinogen 0.719 gm. per 100 c.c. of plasma.

Prothrombin Test.

Oxalated plasma.	$CaCl_2$, 1 per cent.	Coagulation.
5 drops	1 drop	11 min.
5 drops	2 drops	15 min.
5 drops	3 drops	15 min.
5 drops	4 drops	15 min.

Antithrombin Test.[5]

Heated plasma.	Thrombin.	Time interval.	Fibrinogen solution.	Coagulation.
1 drop	3 drops	15 min.	7 drops	15 min.
1 drop	4 drops	15 min.	7 drops	5 min.
1 drop	5 drops	15 min.	7 drops	5 min.

Benzol 2 c.c. given subcutaneously.

[4] Control solutions of fibrinogen and thrombin clotted in from two to four minutes.

[5] Control 2.5 to 3 min.

Nov. 12, 13, and 14. On each of these dates 2 c.c. of benzol were injected. Following the injections the animal became dull, drowsy, and inactive.

Nov. 15. Weight 1,350 gm.; red blood count 4,700,000; white blood count 3,200; platelets 480,000; fibrinogen 0.656 gm. per 100 c.c. of plasma.

Prothrombin Test.

Oxalated plasma.	$CaCl_2$, 1 per cent.	Coagulation.
5 drops	1 drop	40 min.
5 drops	2 drops	31 min.
5 drops	3 drops	30 min.
5 drops	4 drops	38 min.

Antithrombin Test.[6]

Heated plasma.	Thrombin.	Time interval.	Fibrinogen solution.	Coagulation.
1 drop	3 drops	15 min.	7 drops	No clot in 1 hr.
1 drop	4 drops	15 min.	7 drops	6 min.
1 drop	5 drops	15 min.	7 drops	8 min.

Nov. 16, 17, and 18. Daily injections of benzol amounting to 4 c.c. Animal is beginning to look very ill; very weak; does not eat. No paralysis of legs noted.

Nov. 19. Weight 1,175 gm. Red blood count 3,710,000; white blood count 1,900; platelets 185,000; fibrinogen 0.684 gm. per 100 c.c. of plasma.

Prothrombin Test.

Oxalated plasma.	$CaCl_2$, 1 per cent.	Coagulation.
5 drops	1 drop	60 min.
5 drops	2 drops	60 min.
5 drops	3 drops	60 min.
5 drops	4 drops	60 min.

Antithrombin Test.[7]

Heated plasma.	Thrombin.	Time interval.	Fibrinogen solution.	Coagulation.
1 drop	3 drops	15 min.	7 drops	23 min.
1 drop	4 drops	15 min.	7 drops	7 min.
1 drop	5 drops	15 min.	7 drops	7 min.

Benzol 1 c.c. given subcutaneously.

Nov. 20, 21, 22, 23, and 24. Benzol 8 c.c. injected during these five days. Animal becoming very weak and emaciated.

Nov. 25. Weight 1,100 gm. Red blood count 1,810,000; white blood count 500; platelets 128,000; fibrinogen 0.638 gm. per 100 c.c. of plasma.

Prothrombin Test.

Oxalated plasma.	$CaCl_2$, 1 per cent.	Coagulation.
5 drops	1 drop	50 min.
5 drops	2 drops	55 min.
5 drops	3 drops	65 min.
5 drops	4 drops	95 min.

[6] Control 2.5 min.

[7] Control 3.5 min.

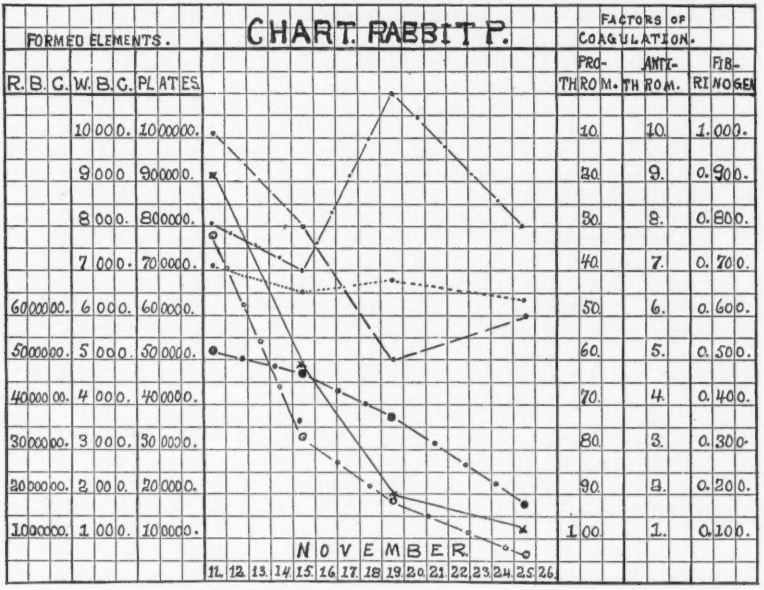
Antithrombin Test.[8]

Heated plasma.	Thrombin.	Time interval.	Fibrinogen solution.	Coagulation.
1 drop	3 drops	15 min.	7 drops	5 min.
1 drop	4 drops	15 min.	7 drops	12 min.
1 drop	5 drops	15 min.	7 drops	5 min.

Nov. 26. Animal killed and autopsied at once. Serous cavities were all normal except for a small organized clot in the pericardial sac. The heart showed many small scars covered with fibrin at the points where the cardiac muscle was pierced in obtaining blood. No gross changes were demonstrable in any of the other organs. Bone marrow obtained from the middle of the femur was mottled brown, of fair consistency, with small pin-point hemorrhages.

Microscopical sections showed an aplastic marrow in the stage of fairly advanced regeneration. In some parts of the section the aplasia was found to be fairly complete, but superimposed upon the edematous aplastic reticulum there were groups and clumps of parenchymal cells; these consisted of granulocytes and normoblasts. Megakaryocytes were present in fairly large numbers.

Reference to the protocol and to table I and text-figure 1 will



TEXT-FIG. 1. Formed elements: heavy dots and dashes = red blood corpuscles; open circles and dashes = white blood corpuscles; solid line = blood platelets. Factors of coagulation: broken line = prothrombin; small dots and dashes = antithrombin; dotted line = fibrinogen.

[8] Control 3 min.

show the effect of benzol poisoning upon both the formed elements and the factors of coagulation in this particular animal. The observations, in this instance, were carried on over a period of two weeks. At the outset the animal was healthy, active, and weighed about 1.5 kilograms. Examinations of the formed elements and the factors of coagulation made at the beginning of the experiment (November 11) showed normal figures. It may be said, however, that the platelet count represents the high limit of normal, and that this is true also of the blood fibrinogen. Benzol injections were given daily, the average dose being two cubic centimeters. Only after four such injections were given did the animal begin to manifest any signs of intoxication. Studies of the blood at this time showed about 50 per cent. reduction in the blood platelets, a similar reduction in the white cells, a moderate grade of anemia, a prothrombin content (as measured by the coagulation time of the recalcified plasma) one-third of the control, an unaltered antithrombin content, and a very slight reduction in the blood fibrinogen.

As the benzol injections were continued, the animal began to exhibit more marked loss of weight, emaciation, anorexia, and dullness. No tendency to bleed was observed, however; but, as indicated in the protocol, both the formed elements and the prothrombin showed a still greater reduction at this time. At the end of two weeks, after twenty-three cubic centimeters of benzol had been injected, a final study of the blood was made. The platelets had fallen to 13 per cent. of the normal, and the prothrombin diminished to such an extent that the coagulation time was six times the control. The antithrombin and fibrinogen values were found to show normal variations.

For purposes of brevity the protocol for experiment G is not reproduced here. The results obtained on this animal are recorded in table II. In this experiment, an example of the second group, determinations of the formed elements and coagulation factors were made over a period of six weeks (September 1 to October 11). At the beginning of the experiment all the formed elements and the prothrombin showed normal values. Unfortunately the antithrombin and fibrinogen were not followed until several weeks after the benzol injections were commenced. Throughout the course of the experi-

TABLE II.

Rabbit G.

Date.	Weight in gm.	Dose of benzol in c.c.	Formed elements.			Factors of coagulation.				
						Prothrombin.		Antithrombin.		Fibrinogen in gm. per 100 c.c.
			Platelets.	W. B.C.	R. B. C.	$CaCl_2$	Min.	Thrombin.	Min.	
Sept. 1	2,200			4,500	4,400,000	1	0			
						2	9			
						3	10			
						4	11			
Sept. 2–5		6	840,000 Sept. 2							
Sept. 5	2,040		520,000	2,400	5,640,000	1	0			
						2	17			
						3	14			
						4	45			
Sept. 7–9		4								
Sept. 9	1,800		612,000	5,600	4,430,000	1	25			
						2	22			
						3	25			
						4	17			
Sept. 11		2								
Sept. 13	1,640		216,000	200	5,670,000	1	18			0.925
						2	15			
						3	19			
						4	19			
Sept. 14		2								
Sept. 16	2,085	2	240,000	1,500	5,100,000	1	12	3	45	
						2	9	4	10	
						3	18	5	5	
						4	9			
Sept. 17–18	2,400	2	144,800	1,800	3,470,000					
Sept. 19	2,085		180,000	1,200	2,880,000	1	Lost	3	25	0.900
						2	18	4	7	
						3	18	5	7	
						4	18			
Sept. 22–26	1,890	4								
Oct. 2	1,830	2		1,400	3,730,000	1	13	3	40	0.252
						2	13	4	27	
						3	12	5	10	
						4	13			
Oct. 3–4		4								
Oct. 5	1,795	2	196,000	1,460	3,780,000	1	8	3	No observation	0.273
						2	6	4	20	
						3	8	5	20	
						4	8			
Oct. 6–11	1,425	16	276,000	1,400	4,230,000					

ment, this animal showed unusual resistance to benzol poisoning, and in spite of the development of transient palsy of the right leg, remained fairly healthy. As the intoxication progressed, the plate-

lets reached as low a level as 180,000, the white cells 1,200, and the red cells 2,800,000. Determinations of the coagulation time of the recalcified plasma during this period showed variations between nine and eighteen minutes, the time becoming less than normal (six minutes) toward the end of the experiment. This shows that the prothrombin content of the blood remained normal in spite of the long period of intoxication and the quite well marked drop in the platelets.

The first antithrombin determinations were made two weeks after the experiment began; this factor was followed subsequently, and at the completion of the experiment no change was noted from the initial determination.

The fibrinogen in the blood fell precipitously during the last three weeks of the experiment. From an initial high level of 0.900 of a gram it fell to 0.273 of a gram. At the time of the last determination, the animal was much emaciated, having lost about 400 grams in weight. This is the only instance in which we have observed such an extensive reduction in fibrinogen following benzol poisoning. We are not prepared to explain this, unless it is due to the gradual wasting and emaciation which developed during the long period of intoxication.

At autopsy we were interested to find that this animal showed evidences of a wide-spread regeneration of the bone marrow. Zones of aplasia still were present, but these were small. Parenchymal elements were found scattered diffusely in long strands throughout the edematous reticulum. Granulocytes and normoblasts were present in abundance, and the megakaryocytes were also found in considerable number. The relatively high platelet count, in spite of repeated benzol injections, is in keeping with such a regenerating marrow.

SUMMARY OF RESULTS.

Formed Elements.—By referring to tables III and IV, it will be seen that all the formed elements are reduced materially in the blood of benzolized rabbits, although the leucotoxic action is the most pronounced. In normal rabbits the average leucocyte count is 7,820 with variations between 11,000 and 4,200. With few exceptions benzol injections cause a striking drop in the count. In our series the white cells dropped to an average level of 1,390, with varia-

TABLE III.

Summary. Prothrombin Diminished. Antithrombin and Fibrinogen Normal.

Date.	Rabbit.	Weight in gm.	Total dose of benzol in c.c.	Formed elements.			Factors of coagulation.[9]			Bone marrow.
				Platelets.	W. B. C.	R. B. C.	Prothrombin per min.	Antithrombin per min.	Fibrinogen in gm. per 100 c.c.	
Aug. 15	B		18		4,200	5,400,000	6			Well marked aplasia. Reticulum aplastic and hyperemic; small nests of erythroblasts; megakaryocytes fairly numerous.
Aug. 24					0	5,480,000	35			
Aug. 31	E	1,460	8	760,000	8,400	4,960,000	9			Advanced aplasia. Edema and hyperemia of reticulum; practically no parenchymal elements remaining, excepting fixed tissue cells, lymphocytes, and polyblasts.
Sept. 5		1,420		520,000	3,100	4,430,000	14			
Sept. 10		1,200		148,000	900	3,930,000	310			
Oct. 2	K	1,380	12		8,400	5,980,000	8	27	0.587	Well marked aplasia (compare rabbit B).
Oct. 5		1,305		408,000	5,200	3,980,000	8		0.679	
Oct. 7		1,300		502,000	11,200	4,200,000	18	23	0.639	
Oct. 10		1,160		336,000	20,800	2,130,000	34	15	0.899	
Oct. 2	L	1,620	18		6,400	4,200,000	13	31		Advanced aplasia. Extreme hyperemia (compare rabbit E).
Oct. 5		1,550		720,000	4,600	3,800,000	10	35	0.543	
Oct. 11		1,205		364,000	2,200	3,000,000	24	19	0.702	
Nov. 11	N	1,300	23	780,000	10,400	4,100,000	10	12	Lost	Advanced aplasia. Almost complete disappearance of parenchymal elements (compare rabbits E and L).
Nov. 15		1,300		320,000	3,000	4,070,000	19	9	0.532	
Nov. 19		1,122		234,000	1,400	2,800,000	39	11	0.524	
Nov. 25		910		64,800	800	2,300,000	70	17	1.200	
Nov. 11	O	1,460	23	620,000	9,200	5,800,000	15	8	0.510	Advanced aplasia. Microscopic picture identical with that of rabbits L and N.
Nov. 19		1,345		242,800	3,200	3,980,000	39	14	0.438	
Nov. 25		1,170		31,200	900	2,100,000	45	8	0.422	
Nov. 11	P	1,400	23	920,000	7,800	5,200,000	11	8	0.719	Aplastic marrow with early regeneration. Areas of complete aplasia with groups and islands of parenchymal cells, erythroblasts, and granulocytes. Megakaryocytes numerous but poorly preserved.
Nov. 15		1,350		480,000	3,200	4,700,000	30	7	0.656	
Nov. 19		1,175		185,000	1,900	3,710,000	60	11	0.684	
Nov. 25		1,100		128,000	500	1,810,000	50	8	0.638	
Nov. 11	Q	1,640	23	530,000	8,800	5,400,000	12	8		Aplasia with well advanced regeneration. Long strands of parenchymal cells; megakaryocytes, both young and adult forms, in large numbers.
Nov. 15		1,510		524,000	4,700	4,200,000	16	9	0.226	
Nov. 19		1,440		120,000	4,000	4,200,000	30	11	0.368	
Nov. 25		1,470		76,000	980	2,800,000	50	9	0.321	
Dec. 2	R	1,930	14	700,000	11,000	4,200,000	9	19	0.852	Advanced aplasia. Only fixed tissue cells, lymphocytes, and polyblasts present.
Dec. 10		1,600		280,000	2,100	4,400,000	25	16	0.708	
Dec. 12[10]							75	7		

[9] The figures in the prothrombin columns represent the coagulation time in minutes of the tube containing the optimum amount of calcium; the figures in the antithrombin columns represent the average clotting time of the tubes containing 3, 4, and 5 drops of thrombin.

[10] Blood obtained in the usual manner five minutes after death.

TABLE IV.

Summary. Prothrombin, Antithrombin, and Fibrinogen Normal.

Date.	Rabbit.	Weight in gm.	Dose of benzol in c.c.	Formed elements.			Factors of coagulation.			Bone marrow.
				Platelets.	W. B. C.	R. B. C.	Prothrombin per min.	Antithrombin per min.	Fibrinogen in gm. per 100 c.c.	
Sept. 1	F	2,125	15	600,000	6,600	5,110,000	8			Aplasia.[11] Marked edema of reticulum; capillaries filled with red cells; clumps of erythroblasts and polyblasts; megakaryocytes numerous.
Sept. 5		2,110		560,000	3,300	5,640,000	17			
Sept. 11		2,010		312,000	266	3,760,000	8			
Sept. 1	G	2,200	46	840,000	4,500	4,480,000	9			Marrow in state of active regeneration. Large islands of granulocytes separated by small areas of aplastic reticulum; numerous islands of normoblasts inside and outside of capillaries; megakaryocytes fairly abundant.
Sept. 5		2,040		520,000	2,400	5,640,000	14			
Sept. 9		1,800		612,000	5,600	4,430,000	17			
Sept. 13		1,640		216,000	200	5,670,000	18		0.925	
Sept. 16		2,080		240,000	1,500	5,100,000	9	20		
Sept. 19		2,025		180,000	1,200	2,800,000	18	13	0.900	
Oct. 2		1,830			1,400	3,730,000	13	25	0.252	
Oct. 5		1,795		196,000	1,460	3,780,000	6	20	0.273	
Sept. 14	H	1,600	4	744,000	6,600	6,400,000	11	10	0.716	Hyperplasia of marrow. Parenchymal cells in dense masses and well preserved.
Sept. 16		1,500		254,000	10,200	4,550,000	15	12		
Sept. 14	I	2,375	6	460,000	7,800	5,410,000	13	9	0.123	No sections.
Sept. 19		2,205		280,000	2,800	5,090,000	16	15	0.240	
Dec. 2	U	1,510	14	650,000	8,000	4,900,000	10	7	0.432	Regenerating marrow (compare rabbit G).
Dec. 10		1,200		570,000	3,800	3,700,000	20	11	0.768	
Dec. 2	T	1,730	42	840,000	9,200	5,600,000	9	8	0.252	Hyperplasia of marrow (compare rabbit H). Normal architecture preserved; no areas of aplasia; cellular elements increased in number; granulocytes and erythroblasts well preserved; megakaryocytes numerous, and of young and old type.
Dec. 10		1,600		412,000	6,300	4,900,000	20	6	0.211	
Dec. 14		1,550		253,000	7,200	3,940,000	20	6	0.260	
Jan. 8		1,470		328,000	2,000	2,800,000	9	5	0.728	

[11] Death occurred five days after the last prothrombin determination. On the day before death platelets were 208,000; white blood count 600; and red blood count 2,730,000.

tions between 3,800 and the complete disappearance of leucocytic elements from the circulating stream. The extent of reduction depends upon the susceptibility of the animal. We have records of only two instances in which the intoxication was associated with a leucocytosis. In one of these (experiment K), after an initial fall, the white cells rose rapidly to 20,800; autopsy gave no satisfactory explanation of this leucocytosis. In the second observation (experiment H) the leucocyte count rose forty-eight hours after the first injection. This may be explained by the initial stimulating action of the benzol.

Some reduction in the number of platelets of the circulating blood is invariably the rule. The fall in count, however, is not usually so pronounced, in most instances, as that of the leucocytes. The average platelet count in normal rabbits was found to be 683,400, with variations between 920,000 and 408,000. These averages approximate those recorded by Duke (4) (average 757,000, with variations between 1,200,000 and 380,000). Following benzol inoculations wide variations in the extent of platelet reduction occur. In our series the average number of platelets following benzol injections was 233,800. Only once were we successful in lowering the count to 31,000. This fact explains our inability to produce in these animals hemorrhagic symptoms, such as occur only after more extreme lowering of the platelet count.

Where the platelets and leucocytes were found much diminished in number, the marrow usually showed fairly well marked aplasia. It should be emphasized, however, that destructive changes rarely were noted without signs of regeneration. In some specimens megakaryocytes could hardly be found; whereas in others giant-cells were present in enormous numbers (experiment Q). In the latter case it was apparent that regeneration was going on at a very rapid rate. It is not unlikely that such rapid regenerative phenomena offer some explanation for the difficulty encountered in reducing the blood platelets in rabbits to a dangerously low level.

Although the erythroblastic tissue of the bone marrow suffers, the circulating erythrocytes are injured relatively little. From an average cell count of 5,100,000 the erythrocytes in our series fell to an average level of 3,500,000 following benzol injections. The lowest

reduction in number was obtained in experiment P, in which the red cells were reduced to 1,800,000. As marked an anemia as Selling noted in his clinical cases of benzol poisoning (640,000 and 1,150,000 red cells) apparently cannot be reproduced experimentally in this way.

Antithrombin.—Determinations of the amount of antithrombin in the blood were made in twelve of the fifteen experiments. Great care was exercised to keep the technique uniform throughout the series, and in seven of the experiments the same strengths of thrombin and fibrinogen solutions were used, so that these latter are comparable among themselves. In none of the examinations was the variation from the control found to be large. Of the twelve complete records, about six (experiments G, H, O, P, Q, and T) showed an unaltered antithrombin content following benzol inoculations; three (experiments K, L, R) showed a slightly diminished, and three (experiments N, I, and U) a slightly increased amount. The slight diminution or excess observed in some instances depends probably upon the period during which the observation was made. It will be seen from text-figure 1 (rabbit P) that there was an interval during the experiment (November 19) when the antithrombin of the blood was present in excess (compare also experiments G, L, and O). Such fluctuations in the relative amount of antithrombin are due most likely to variations in the supply of tissue juices (thromboplastin), for it will be recalled that during the normal circulation, platelets are continually undergoing dissolution, and that the thromboplastin which they furnish is capable of neutralizing antithrombin.

Prothrombin.—The relative amounts of prothrombin, before and after benzol injections, were determined by the method already described. It may be said, in general, that the time of coagulation of the recalcified plasma of normal rabbits was fairly constant, averaging about ten minutes, with variations between six and fifteen minutes. The average coagulation time of such plasma of benzolized rabbits was found to be about fifty minutes, although, as will appear from table III, wide variations of between twenty-four and three hundred and ten minutes were observed. It is of interest that in the latter experiment, which represents an isolated instance where the coagulation time was so markedly prolonged, the marrow

at autopsy showed the most complete aplasia observed in the entire series.

The delayed coagulation of the recalcified plasma of benzolized rabbits may be explained theoretically in one of two ways: it may be due, in the first place, to the presence of an excessive amount of antithrombin; or, in the second place, to a deficiency of prothrombin. In the preceding paragraphs it has been shown, however, that the amount of antithrombin is not altered materially by benzol injections; in fact, there may be relative diminution in the amount, so that the prothrombin deficiency may be even greater than the recorded coagulation time indicates. It is fair to assume, therefore, that the delay in the coagulation time of the recalcified plasma is due to an actual deficiency of prothrombin, and not to an excess of antithrombin.

An examination of table III will show, furthermore, that this prothrombin deficiency does not parallel absolutely the reduction in blood platelets. For instance, certain animals with a much lower count may show a less marked diminution in prothrombin than animals with a higher count (compare experiments E, P, and U with O and Q). Such findings are readily explainable, however, if it is kept in mind that regeneration of the myeloid tissue usually goes hand in hand with aplasia, and that the number of platelets at any given time will depend largely upon the degree of regeneration. This fact helps also to explain why such difficulty is encountered in lowering both the platelets and the prothrombin of the circulating blood to a dangerous point.

No definite parallelism was demonstrable between the prothrombin deficiency and the reduction in leucocytes. The white cells may be much reduced or absent at a time when the prothrombin content is little altered (experiments F and G), or there may be some deficiency in prothrombin associated with a leucocytosis (experiment K). In general, however, a prothrombin deficiency has been found associated with a reduction in the number both of platelets and leucocytes.

Fibrinogen.—All the fibrinogen determinations were made by the method of heat coagulation. In every instance the reading was corrected for the oxalate solution used in receiving the blood, so that

the results represent the quantity of fibrinogen in 100 cubic centimeters of actual plasma. Wide fluctuations in the blood fibrinogen of normal rabbits were observed. The average was 0.560 of a gram per 100 cubic centimeters of plasma, but variations between a minimum of 0.123 of a gram, and a maximum of 0.925 of a gram were noted. This is in harmony with Whipple's figures for normal healthy dogs (average 0.466 of a gram; minimum 0.198 of a gram; maximum 0.867 of a gram) (13). In almost all instances repeated injections of benzol (four to forty-two cubic centimeters) caused a rise in the blood fibrinogen. The average of the final readings made from two to thirty days after the initial determination was found to be 0.617 of a gram per 100 cubic centimeters of plasma, but in some individual observations enormous rises were observed (experiments I, K, L, N, Q, T, and U). In about three experiments (O, P, and R) little variation from the normal occurred, and in only one instance (experiment G) was there a striking reduction in the blood fibrinogen. A possible explanation for this drop has been found in the marked loss of weight and extreme cachexia produced in this animal by the benzol inoculations. Similar reductions in the quantity of fibrinogen have been reported by Whipple in human cases of cachexia. The low values in such cases are difficult to explain.

The rise in blood fibrinogen observed in the majority of animals following benzol injections has been observed also in other conditions associated with intoxication.[12] It is possible that benzol may stimulate the fibrinogen-forming organs to over-functional activity and that the fibrinogen may rise above normal at these times. Such an increase is not out of keeping with the gross anatomical and the histological appearance of the liver in benzol poisoning. Aside from a slight degree of parenchymatous change, no other pathological lesion was demonstrable in the liver parenchyma.

THE RELATION OF THE BONE MARROW TO THE ORIGIN OF PROTHROMBIN, ANTITHROMBIN, AND FIBRINOGEN.

The experiments and observations already detailed throw some additional light upon this more complex problem of coagulation. In-

[12] Whipple, *loc. cit.* (13), p. 57.

terest in the origin of the various substances concerned in the clotting of blood has stimulated much work among investigators in this field. From time to time different tissues and organs have been made responsible for the elaboration of the factors participating in the process; and, although there are still some workers (14, 15, 16) who ascribe to the liver the important function of forming all the fibrin factors, the majority are agreed that certain of the formed elements of the blood play an important part in the origin of some of these substances. Without entering upon a discussion of the experimental basis for their views, we wish to review briefly some of the work of different observers which is or is not in harmony with our deductions.[13]

Origin of Prothrombin.—With the exception of Wooldridge[14] all workers since Alexander Schmidt (17) have recognized the importance of the formed elements of the blood in the elaboration of prothrombin. The view, which originated with Schmidt, that the leucocytes give rise to this fibrin factor has been discredited largely by later work (11, 18). Bizzozero (19) was the first to suggest the platelets as the possible prothrombin formers. To him, too, we owe the knowledge that these elements are independent morphological structures and not degeneration products of leucocytes or erythrocytes. The work of Morawitz (18) has furnished the necessary experimental evidence that the theory of Bizzozero and others was correct. Both Morawitz and more recently Bayne-Jones (20), working in Howell's laboratory, have demonstrated conclusively that aqueous extracts of platelets obtained by differential centrifugalization contain a substance which will clot fibrinogen solutions in the presence of calcium. They have shown further that platelets contain another substance, thromboplastin, so that the disintegration and solution of platelets, when blood is shed, facilitate clotting in two ways: first, by setting free prothrombin; second, by liberating a thromboplastic substance which hastens coagulation by neutralizing the antithrombin present normally in circulating blood.

Origin of Antithrombin.—It has long been known that the intravenous injection of peptone solutions into animals gives rise in the blood of these animals to a substance which is capable of inhibiting coagulation (Schmidt-Mülheim, 1880). The French School, in particular, has done much experimental work to learn in what organ and by what mechanism this anticoagulation substance is formed. The work of Contjean (21), Delezenne (22), and others has established with certainty that the liver is essential for the formation of this substance, and that antithrombin is not formed if the liver be excluded. There is no direct evidence, however, that this coagulation-inhibiting substance is of the same nature as the antithrombin present normally, in greater or less amount, in

[13] Excellent discussions of this phase of the subject will be found in the papers by Morawitz (prothrombin), Delezenne (antithrombin), and Whipple and Hurwitz and Meek (fibrinogen).

[14] Cited by Morawitz (18).

the blood of animals and man. Their identity is assumed because they both act in a similar way. In this connection it is of interest to note that Whipple (23), in a study of clinical cases, observed fluctuations in antithrombin in the blood of patients suffering from liver disease and in one patient with aplastic anemia. In the latter instance antithrombin was present in excess, although the marrow was completely aplastic. It would appear, therefore, that the reaction of the bone marrow is not a factor in the production of antithrombin. This observation is in harmony with the conclusion deduced from our experiments; namely, that aplasia of the marrow in rabbits produces no appreciable change in the antithrombin of the blood.

Origin of Fibrinogen.—An extensive discussion concerning the origin of blood fibrinogen is not needed, as this phase of the subject has been treated fully in several recent papers (13, 24). Suffice it to say that various workers have adduced evidence in support of one or other of the following theories regarding its origin: first, that fibrinogen is formed by the intestine (Mathews (25)); second, that fibrinogen is either produced in the liver or is wholly dependent upon that organ for its production (Doyon (26) and his coworkers, Nolf (27), Whipple and Hurwitz (12), and Meek (24)); third, that fibrinogen is formed by the bone marrow and is perhaps dependent upon the white cells (Müller (28), Morawitz and Rehn (29)).

It would appear that the best experimental evidence is in favor of the second view, although recent studies (Goodpasture (30)) on the regeneration of fibrinogen show that normal fibrinogen production is a result of the combined activity of the liver and the intestine, the latter organ being an important contributing factor in the rapid formation of fibrinogen.

As far as the authors are aware, no direct experimental proof has been produced for or against the view of Müller and of Morawitz and Rehn that the bone marrow is a fibrinogen former. Only a few casual observations are recorded by recent workers upon this function of the marrow. Whipple and Hurwitz[15] noted that with the rapid drop in fibrinogen following chloroform poisoning, there may be a leucocytosis caused by the liver necrosis and repair, and that the bone marrow at such a time may be hyperplastic. Conversely, Whipple,[16] in a study of a clinical case of aplastic anemia, has shown the blood fibrinogen to be normal (0.4032 of a gram), whereas the bone marrow at autopsy was found to be completely aplastic. Similarly in our observations upon experimental aplastic anemia in benzolized rabbits, we have seldom noted low fibrinogen values associated with aplasia of the bone marrow. In almost all our observations the fibrinogen was found either little changed from the normal or somewhat higher than normal. In only one experiment (G) was there any appreciable drop in fibrinogen, and we believe that this has been satisfactorily explained. It seems fair to conclude from such evidence that the bone marrow does not participate in the production of blood fibrinogen.

[15] Whipple and Hurwitz, *loc. cit.*, p. 138.

[16] Whipple, *loc. cit.* (13), p. 59.

DISCUSSION.

The experimental observations recorded have shown that injury to the myeloid tissue by benzol causes a diminution in the prothrombin content of the blood, and that this tissue must be intimately associated with the elaboration of this substance. Our experiments offer no convincing evidence, however, as to the relative importance of the various formed elements of the marrow in prothrombin production; but the more direct proof given by other workers makes it fairly certain that the platelets and not the leucocytes are the essential elements. Neither Morawitz (18) nor Howell (11) have been able to obtain satisfactory proof that the white cells contain any appreciable amount of prothrombin, whereas both workers have prepared active prothrombin solutions from aqueous extracts of blood platelets. In view of these observations the conclusion appears justified that the blood platelets play more than a mere mechanical part in the process of coagulation. Presumably, during the normal circulation, these elements undergo more or less gradual dissolution and thus help to maintain the prothrombin equilibrium of circulating blood.

Already attention has been directed to the lack of parallelism between the drop in prothrombin and the extent of destruction of myeloid tissue. This finding is in keeping with the experimental and clinical observations of other workers. Pratt (31), for instance, has shown that there is no direct interdependence between the number of platelets and the coagulation time. More recently Duke has emphasized this point by showing that blood in which the platelets are reduced to 10 per cent. of normal by repeated benzol injections may clot at a normal rate. Undoubtedly in such extreme cases the delayed bleeding time must depend upon the mechanical importance of these elements, for it is well known that agglutination of platelets is essential to effective clotting. It follows, therefore, that the platelet count and the circulating prothrombin may be considerably diminished without causing symptoms of hemophilia.

On the whole, the conservative conclusion would seem to be that the maintenance of the prothrombin equilibrium of the blood depends only in part upon the blood platelets. Besides the facts already mentioned, there is other evidence in support of this view. It is well established, for instance, that the platelet count may be

perfectly normal in hemophilia in which the coagulation time may be markedly prolonged in consequence of a deficiency in the amount of the contained prothrombin (7). Furthermore, in a recent publication, Howell (11) has shown that prothrombin is present in solution in the circulating lymph, although blood platelets do not constitute a normal element of this fluid. The delayed clotting of lymph depends rather upon a deficiency in the contained thromboplastin, and consequently upon a relative excess of antithrombin. Such evidence suggests the possibility that some tissue in addition to the bone marrow is concerned with prothrombin formation. This view is supported also by the knowledge, to which reference has already been made, that the elaboration of fibrinogen depends upon the combined activity of at least two organs,—liver and intestine. The maintenance of the prothrombin equilibrium of the blood is obviously so important for the organism that the assumption that other tissues or organs participate in this process harmonizes better with our present conception of the factors of safety in physiological processes emphasized by Meltzer.

Our experiments afford no support for or against the view of Nolf and others that liver cell activity is essential for prothrombin production, nor do they make it clear what tissues besides the marrow play a part in its elaboration.

CONCLUSIONS.

1. Subcutaneous injections of benzol in rabbits produce marked destructive changes in the hematopoietic organs, especially in the myeloid tissue.

2. Benzol poisoning registers a change not only in the formed elements of the blood, but also in the factors of coagulation.

3. The circulating prothrombin is considerably reduced in amount and in most instances animals in which such a diminution occurs show aplasia of the bone marrow.

4. The association of extreme aplasia of the marrow without a fatal diminution in the circulating prothrombin suggests one of two possibilities: either other tissues and organs in addition to the bone marrow are concerned with prothrombin formation; or a minimum amount of myeloid tissue suffices to maintain the quantity of prothrombin above a dangerous level.

5. The myeloid tissue plays no part in the production of antithrombin.

6. Bone marrow activity is not essential for the production of fibrinogen.

In conclusion we desire to express our appreciation to Dr. Katherine R. Drinker for numerous fibrinogen determinations, and to the Pathological Department of the Peter Bent Brigham Hospital for many courtesies.

BIBLIOGRAPHY.

1. Drinker, C. K., and Hurwitz, S. H., *Arch. Int. Med.*, 1915, xv (in press).
2. Selling, L., *Bull. Johns Hopkins Hosp.*, 1910, xxi, 33.
3. Selling, *Johns Hopkins Hosp. Rep., Monographs*, 1913, No. 2.
4. Duke, W. W., *Arch. Int. Med.*, 1913, xi, 100.
5. Duke, *Jour. Am. Med. Assn.*, 1910, lv, 1185; *Jour. Exper. Med.*, 1911, xiv, 265.
6. Wright, J. H., *Boston Med. and Surg. Jour.*, 1906, cliv, 643.
7. Howell, W. H., *Arch. Int. Med.*, 1914, xiii, 76.
8. Howell, *Am. Jour. Physiol.*, 1910, xxvi, 453; 1911–12, xxix, 187; 1912–13, xxxi, 1; 1913, xxxii, 264; 1914, xxxv, 474.
9. Wright, J. H., and Kinnicutt, R., *Jour. Am. Med. Assn.*, 1911, lvi, 1457.
10. Lee, R. I., and Vincent, B., *Arch. Int. Med.*, 1914, xiii, 398.
11. Howell, *Am. Jour. Physiol.*, 1914, *loc. cit.*
12. Whipple, G. H., and Hurwitz, S. H., *Jour. Exper. Med.*, 1911, xiii, 136.
13. Whipple, G. H., *Am. Jour. Physiol.*, 1914, xxxiii, 50.
14. Nolf, P., *Ergebn. d. inn. Med.*, 1913, x, 275.
15. Corin, G., and Ansiaux, G., *Jahresber. ü. d. Fortschr. d. Thierchem.*, 1894, xxiv, 642.
16. Loeb, L., *Med. News*, 1905, lxxxvi, 577.
17. Schmidt, A., Zur Blutlehre, Leipzig, 1892.
18. Morawitz, P., *Deutsch. Arch. f. klin. Med.*, 1904, lxxix, 215.
19. Bizzozero, J., *Virchows Arch. f. path. Anat.*, 1882, xc, 261; cited by Morawitz, *loc. cit.*, p. 216.
20. Bayne-Jones, S., *Am. Jour. Physiol.*, 1912, xxx, 74.
21. Contjean, C., *Arch. de physiol. norm. et path.*, 1895, xxvii, 245.
22. Delezenne, C., *Arch. de physiol. norm. et path.*, 1896, xxviii, 655.
23. Whipple, G. H., *Arch. Int. Med.*, 1913, xii, 637.
24. Meek, W. J., *Am. Jour. Physiol.*, 1912, xxx, 161.
25. Mathews, A., *Am. Jour. Physiol.*, 1899–1900, iii, 53.
26. Doyon and Kareff, N., *Compt. rend. Soc. de biol.*, 1904, lvi, 612.
27. Nolf, P., *Arch. internat. de physiol.*, 1905–06, iii, 1.
28. Müller, P. T., *Beitr. z. chem. Phys. u. Path.*, 1905, vi, 454.
29. Morawitz, P., and Rehn, E., *Arch. f. exper. Path. u. Pharmakol.*, 1907–08, lviii, 141.
30. Goodpasture, E. W., *Am. Jour. Physiol.*, 1914, xxxiii, 70.
31. Pratt, J. H., *Arch. f. exper. Path. u. Pharmakol.*, 1902–03, xlix, 299.

THE HISTOGENESIS OF CHRONIC URANIUM NEPHRITIS WITH ESPECIAL REFERENCE TO EPITHELIAL REGENERATION.*

By JEAN OLIVER, M.D.

(From the Pathological Laboratory of the Medical School of Leland Stanford Junior University, San Francisco.)

PLATES 23 TO 30.

INTRODUCTION.

Since the appearance of Dickson's (1) original work on the production of chronic nephritis by the administration of uranium nitrate, comparatively little morphological investigation has been done on this subject. With the exception of Christian (2) and Suzuki (3), investigators have turned their attention to the functional study of uranium nephritis, especially in its acute stages, and to the related condition of edema formation. Among those who have studied the former subject may be noted Folin (4), MacNider (5), and Christian (6); while the latter question has interested Richter (7), Schlayer and Hedinger (8), Georgopulos (9), and, in this country, Pearce (10). The results of these studies have cast much light not only upon the effect of the renal lesion on the function of the kidney, but have also suggested a possible explanation for the progression of acute uranium lesions into chronic processes.

It has long been known that there is little or no tendency towards the production of a progressive chronic nephritis after the acute degenerative lesions following most of the renal poisons, whereas the development of such a progressive chronic nephritis is constant after acute lesions produced by uranium. Ophüls (11) has shown that there is no such tendency to the formation of a chronic nephropathy after acute chromium nephritis, a fact which is generally admitted by those who have studied the action of the different toxic agents (Suzuki (3)). It is true that slight interstitial lesions have been described by some writers, following the administration of chromium salts (Kabierske (12), Pander (13), and Smith (14)), but there is never the marked connective tissue proliferation with subsequent shrinkage which is so regularly seen in uranium poisoning.

The hypothesis advanced by Dickson to explain this fact held that there was a stimulative irritation of the connective tissue elements coincident with the destruction of the epithelium. This view was supported by the progressive nature of the connective tissue proliferation, its development around the blood vessels,

* Aided by a grant from The Rockefeller Institute for Medical Research, under the direction of Dr. E. C. Dickson. Received for publication, February 8, 1915.

and by the previously mentioned functional work which showed uranium to be a vascular as well as an epithelial poison.

Ophüls (15) in discussing the relation of acute epithelial degenerations to connective tissue proliferation says:

If any irritant acts simultaneously on both the epithelium and the connective tissue this (*i. e.*, early epithelial degeneration and latter connective tissue proliferation) must necessarily happen. The epithelial changes are early because the degeneration can and does occur within a short time, whereas the more slowly developing changes in the connective tissue show much later, necessarily. . . . Moreover, it is plainly brought out by all experimental evidence on this question that the more chronic the process the less marked the lesions in the epithelium and the more pronounced the interstitial changes. . . . I believe, therefore, that, on the contrary, the experimental evidence furnishes almost positive proof that the changes in the epithelium and the connective tissue are coördinate, not subordinate, to one another. They are the effect of the same cause acting simultaneously on different tissues, with different vulnerability and different reaction time, so far as visible changes are concerned.[1]

The opposite view of the relation of the two processes has been taken by Aschoff (16) and his pupil Suzuki. They made use of vital staining in an extensive study of the morphology of the renal secretion under normal and abnormal conditions, such as in the acute nephritides following different renal poisons, and showed that when uranium is injected in moderate doses it affects mainly the terminal part of the proximal convoluted tubule (*"Hauptstück"*), and so differs in its action from chromium, corrosive sublimate, arsenic, and cantharidin, which affect other parts of the same segment of the renal tubule. In chronic uranium nephritis, which they found regularly after the acute lesion, the connective tissue proliferation starts in that region where the epithelial damage was greatest, *i. e.*, in the outer stripe of the outer zone of the medulla (Peter (17)), for there the terminal divisions of the proximal convoluted tubules lie. They compare this process with the connective tissue proliferation observed in hydronephrotic atrophy of the kidney. This analogy they base on the assumption that regeneration of the renal epithelium proceeds from the upper part of the descending loop of Henle, which part is destroyed by the action of the uranium, and that the proliferation of the epithelial cells is hindered by the necrotic masses which fill the tubule at this point. They believe that there is a consequent collapse of the tubule in this region, with resulting atrophy of inactivity of the tubule above, and that the connective tissue, though primarily unaffected, proliferates and fills in the dead space.

These contentions of Aschoff and Suzuki all hinge on the exact mode of regeneration of the epithelium, as is evident from the fact that Suzuki's hypothesis is based upon the assumption that regeneration occurs chiefly in the upper portion of the descending limb of Henle; and still a review of the literature shows that remarkably little investigation has been done on this subject either before their work, or as a consequence of it. Beyond the casual statement that regeneration occurs after an acute nephritis, either spontaneous or experimentally produced, little detail is given of the process. Experimental investigations have

[1] Ophüls, W., *loc. cit.* (15), p. 489.

been largely confined to regeneration following mechanical insults, such as puncture, cauterization, ligation of the renal artery, or freezing the cortex. Podwyssozki (18) seems to have been the pioneer in this work, and the studies of Ribbert (19) and Hochhaus (20) have also dealt with the reparative processes in kidneys so treated. It is at once evident, however, that the results of such a gross attack on the renal elements can be applied only to a limited extent to the regeneration following the production of lesions by toxic agents which reach the kidney through the blood stream.

The only extensive study of regeneration following the administration of renal poisons has been made by Thorel (21), although Cornil and Toupet (22) have given a brief description of the mitoses which they observed in the late stages of cantharidin nephritis. Thorel made a systematic study of the reparative changes which occurred during the first nine days of chromium poisoning, and the summary of his results will be discussed during the review of the findings in the experiments which we have performed.

The descriptions of regeneration in human nephritides are of interest, especially in those cases where the newly formed cells were not of normal morphology, as it will be seen that similar conditions are found in the regenerated epithelial cells following uranium nephritis. Thorel (23) has noted atypical mitoses, hypo- and hyperchromatic figures, and the resulting production of a markedly atypical epithelium in a case of secondary contracted kidney; and Rössle (24), Tilp (25), and Oertel (26) have described the formation of giant-cell-like complexes in chronic human nephritis. The work of Heineke (27) should also be mentioned, as it gives a remarkable series of cases of corrosive sublimate poisoning in man, ranging from those in whom death occurred in a few hours, to those who survived for several weeks.

A new method of investigation of experimental nephritis has been afforded by the recent advances in vital staining. While no attempt will be made to review the general literature of this extensive subject, a brief resumé of its application to the study of renal morphology, both normal and pathological, will be given. A more detailed discussion is contained in the monograph of Policard (28).

The earlier studies on the excretion of dyes by the kidney, especially of carmine, were made by Chrzonszczewsky (29) and Ribbert (30). In these investigations, as well as in the articles of the writers who followed them, the carmine was described as being excreted in the convoluted tubules, proximal and distal, and in the ascending loop of Henle. This was the accepted interpretation until the work of Suzuki (3) showed that excretion, or, better, "*Speicherung*," was confined to the proximal convoluted tubule, and did not occur to any extent in any other part of the renal tubule. Suzuki also showed that the proximal convoluted tubule could be divided by means of the amount of vitally stained granules which its different divisions contain, into a proximal division adjacent to the glomerulus where the granules are numerous and arranged in the form of rods; a middle division in which the granules are much fewer in number, but are still arranged in rod-like formations; and lastly a terminal division in which there are but few granules scattered irregularly throughout the renal cells. This last division corresponds roughly with the "*Spiralrohr*" of earlier writers, and can be further differentiated from the ascending loop of Henle whose diameter and epithelium are very similar, by the possession of a cuticular seam, the "*Bürstenbesatz*."

Peter's work (17) has shown that the "*Spiralröhre*" ("*partie terminale*" of Policard) lie in the same region of the kidney as the ascending loops of Henle, *i. e.*, in the outer stripe of the outer zone of the medulla, and that owing to the similarity of their epithelium and the difficulty of properly preserving the delicate cuticular seam, these two divisions of the renal tubule are easily confounded.

Suzuki's application of this method to the various experimental nephritides revealed the fact that the different toxic agents have a selective action on the different parts of the proximal convoluted tubule. For instance, chromium salts affect the heavily granulated division I, uranium affects the terminal sparsely granulated division III, and to a slight extent the moderately granulated division II, while sublimate and cantharidin affect almost exclusively the terminal division III. More complete details of the action of these toxic agents are described in Suzuki's monograph.

A further aid which the method of vital staining lends to the study of experimental nephritis is the behavior of the newly regenerated epithelial cells to the vital stain. It was first pointed out by Ribbert (31) that the newly regenerated cells do not contain the deposits of carmine which are seen in the original renal cells, and that by the application of vital staining a comparatively easy and certain method is obtained for distinguishing these two classes of cells. As will be seen in the discussion of Thorel's study of regeneration following chromium nephritis, this is often a matter of considerable difficulty with the ordinary methods of histological examination.

In the present study an attempt has been made to follow the development of the nephritis from the earliest acute stage through the successive changes which lead to a marked chronic nephritis. Not only have the changes in the regenerated epithelial elements been studied, but especial attention has been given to the early development of the connective tissue and to the origin of this chronic fibrosis. In addition, the interpretations of Suzuki, especially in so far as they deal with the method of production of the chronic lesions in uranium nephritis, have been the subject of careful analysis.

METHODS.

A combination of the methods of Thorel and Suzuki was used. An attempt was made to produce an acute nephritis of fairly constant severity in a vitally stained animal and to observe the progress of the condition by sacrificing the animals on successive days. Guinea pigs were used for the majority of the experiments for two reasons. First, as Thorel has pointed out, it is necessary to produce the greatest possible damage to the kidney which is compatible with life in order to obtain constant results in the severity of the acute nephritis

in the various animals and in the consequent progress of the condition. Rabbits are much more susceptible to the poisonous action of renal irritants and a large percentage succumb during the acute stage of the intoxication. Moreover, they are much more susceptible to spontaneous chronic nephritis than are guinea pigs (Ophüls (32)), and its possible occurrence is apt to lead to confusion with the experimentally produced lesions. Even with these precautions there is always the individual susceptibility of the animal to be considered, and this can only be avoided by a large series of experiments. In some cases where it was necessary to prolong the life of the animal for two weeks or more, a smaller dose of uranium was given, and in these cases, though the animal apparently recovered from the initial acute attack, it eventually died of the resulting chronic nephritis (compare guinea pig 51, figure 1). In detail the procedure was as follows. The guinea pig received five milligrams of uranium nitrate in a 1 per cent. solution, and on the two following days, two cubic centimeters of a 5 per cent. solution of carmine in saturated lithium carbonate solution. The animals were killed at the proper time, unless death resulted spontaneously, and the kidneys were fixed immediately in 10 per cent. formalin. A brief washing in running water, followed by imbedding in paraffin section, and a light counterstain with hematoxylin, and Van Gieson solution completed the technique. In the later stages, after the tenth day, the animals were sacrificed every other day.

For the purpose of comparison a small series of rabbits was used, which also received five milligrams of uranium but no carmine, and a series of white rats which received uranium, chromium, or sublimate, with subsequent vital staining.

The number of animals used was: guinea pigs receiving uranium, fifty-four; white rats, eighteen, six receiving uranium, chromium, and sublimate, respectively; and twenty-two rabbits which received uranium alone. In order to save space and to avoid repetition, the individual protocols of the animals have been omitted, and only a general description of the process observed in them is given.

THE APPEARANCE OF THE NORMAL KIDNEY IN VITAL STAINING.

But brief mention of our findings in the normal kidney as studied by vital staining will be given, as our results coincide perfectly with those of Suzuki (3). The proximal convoluted tubule alone shows the deposit of carmine granules, and in this region the number of granules decreases as one departs from the glomerulus. In the following descriptions we shall therefore use Suzuki's terminology and refer to the different divisions as divisions I, II, and III, numbering from above downwards, the heavily granuled division with rod formation, the medial lesser granuled division, and the terminal, sparsely granuled division.

The distribution of the vitally stained granules may be followed in serial sections, but a more certain method is that of maceration in hydrochloric acid with subsequent teasing out of the kidney tubules. In kidneys heavily stained with trypan blue the granules will resist the action of the acid if not left too long, and appear a dark green color. Figure 12 shows the glomerulus and the proximal convoluted tubule (*Hauptstück*) with a decreasing number of stained granules, as one approaches the narrow descending limb of the loop of Henle. Figure 13 shows the distal convoluted tubule (A) (*Schaltstück*) connecting with the collecting tubule (B). Though the cells of the former are somewhat more granular than the transparent cells of the collecting tubule, no vitally stained granules are present in them. Similarly no granules are found in either limb of the loop of Henle or in the collecting tubules.

ACUTE NEPHRITIS FOLLOWING VARIOUS TOXIC AGENTS.

(FIRST TO THIRD DAY.)

URANIUM.

Guinea Pigs.—Immediately following a moderate dose of uranium nitrate, five milligrams for a large guinea pig, the lesions observed are striking in constancy and localization. Division I and, as a rule, division II of the proximal convoluted tubule are intact, and the epithelium shows the normal deposit of vitally staining granules. As one approaches division III, the damage to the renal cells becomes evident. The cytoplasm of the renal epithelium is destroyed

and the lumen of the tubule is filled with a granular detritus which is stained a diffuse pink by the carmine. The nuclei are either pycnotic and stained a bright red, or show various stages of disintegration. A few cells persist in which the nuclei are still normal in appearance and in which the cytoplasm shows but a slight amount of change as indicated by irregularities in the arrangement of the vitally staining granules. This persistence of the cells in the degenerated areas must be kept in mind, as it is a point of importance in the later changes in the epithelial structures. The ascending loops of Henle show a slight amount of damage in the more severe cases, perhaps somewhat more commonly in our specimens than Suzuki has described.

It will be seen that the greater part of the damaged tubules lie in the outer stripe of the outer zone of the medulla, a fact which can be recognized in the gross appearance of the kidneys. In rabbits in which the various divisions of the medulla are well marked, this distribution is easily seen as a broad band of deeply vitally stained, opaque degenerated tissue extending between the cortex and the inner stripe of the outer zone of the medulla.

The other divisions of the kidney are practically normal. In the collecting tubules are a varying number of hyaline casts, which, if the injection of carmine has but shortly preceded the death of the animal, are stained with carmine. Very few casts, exclusive of the masses of epithelial detritus, are seen in the proximal convoluted tubules or the loops of Henle.

To sum up, the process is limited to division III of the proximal convoluted tubule, but as the severity of the lesion increases, the damage ascends towards division II, and is also seen in the ascending limb of Henle's loop. A fact of much importance is the persistence of a few cells with normal nuclei and only slightly disturbed cytoplasm. When one considers the small part of the tubule seen in one section, the number of these persisting cells must be considerable.

Rabbits.—The lesion in the rabbit kidney is similar to that observed in guinea pigs except in two respects. The kidney of the rabbit is relatively much more susceptible to all irritants than is that of the guinea pig. The lesions are therefore much more extensive, and with the same dose as that given to the guinea pigs, five milli-

grams, there is necrosis of practically the entire proximal convoluted tubule, as well as marked degeneration in the ascending loop of Henle. These tubules are filled with dense masses of granular detritus, and many casts are seen in the collecting tubules.

Another striking feature is the change seen in the glomeruli of many of the animals. Christian (33) has described various lesions in the glomeruli of uranium rabbits, hyaline droplets in the capillary walls, fibrin thrombi in the capillaries, dilatation of the capsular space with granular material, and proliferative lesions affecting the capillary endothelium. In a comparatively large number of our rabbits, lesions were observed in the glomeruli, consisting as a rule of a filling of the capsular space with granular material, and pycnosis of the endothelial nuclei in the loops.

White Rats.—(Figure 2.) The lesions observed in white rats differed in no way from those observed in guinea pigs. The tendency to repair is great in these animals, and the regenerative changes, though essentially the same, appear more quickly than in guinea pigs and are therefore present before the end of the third day.

CHROMIUM.

(Figure 3.) To confirm Suzuki's description of the marked difference in the action of chromium and uranium on the renal tubule, a few white rats were injected with two milligrams of potassium bichromate followed by carmine.

The difference from the histological picture of acute uranium nephritis is evident at first glance. The heavily stained divisions I are lacking, and their place is occupied by tubules which consist of the bare membrana propria which are filled with a pinkish granular detritus. As one approaches the medulla a few normal vitally stained tubules are seen, and in the outer stripe of the outer zone of the medulla are seen the faintly vitally stained divisions III, practically in normal condition. In severe cases pycnotic nuclei are present even in this last division, but never the severe necrosis observed in uranium poisoning. The glomeruli are normal in all cases.

SUBLIMATE.

The acute sublimate nephritis resembles more nearly that observed after uranium administration. The heavily stained division I is intact, and in all but the most severe cases division II is also. Division III shows a marked necrosis of the epithelium, which extends well down into the medulla. This extension downwards is to a lower level than is ordinarily seen in uranium nephritis, but it is difficult to say whether this is due to the size of the dose employed or to an intrinsic difference in the action of the two toxic agents.

THE REGENERATIVE CHANGES IN THE EPITHELIUM FOLLOWING ACUTE URANIUM NEPHRITIS.

The picture of acute uranium nephritis described above holds for the first three days of the process. It is only at the end of the third day, and in guinea pigs often during the fourth day, that the process of regeneration becomes at all marked. In such preparations one is at once struck by the appearance of the affected tubules. The greater number still contain granular detritus which more or less completely fills the lumen. In many cases the tunica propria is still bare, but in others two or three comparatively enormous nuclei are seen which are covered with a rather indistinct layer of protoplasm. These nuclei, as a rule oval in shape, possess a relatively large amount of deeply staining chromatin, which is arranged in a loose thread-like network, and possess one, and at times two, large nucleoli. The long axis of these nuclei is indifferently arranged in relation to the axis of the tubule, a fact which becomes quite striking in the later stages when a large number of them is present.

In the same tubules in addition to the large nuclei are smaller ones, approximating the size of the normal renal cell or even smaller. Many of these show no detail in their chromatin, but present a dense, and when stained with hematoxylin, a black appearance. In preparations where the vital staining has been especially intense, the cytoplasm shows a few scattered carmine granules. There can be no doubt that in these we have to do with the original renal cells whose nuclei have been severely affected by the uranium, but which still retain a certain amount of vitality. They correspond to the cells which Thorel has described in various degenerative processes and which he believes are evidences of early regeneration.

In the neighborhood of these large newly formed nuclei one can often find mitotic figures in varying stages of division. The number of mitoses is always small as compared with the number of the regenerated cells, a fact which has been emphasized by many writers. The spindle figures seen are entirely normal, there being no evidences of asymmetrical distribution of the chromatin (Thorel (23)).

Besides these spindle figures, we would call especial attention to the premitotic and early stages of the process of mitosis. These have not been described in detail by other writers, and if one considers their number in addition to the spindle forms, the discrepancy between the number of mitotic figures and the active nature of the regenerative process is much reduced. The preliminary changes which the nucleus commonly undergoes prior to the process of indirect division are all well shown in the regenerating tubules. One of the commonest observations is the enlargement of the nucleus, with the formation of a loose spirem. In other nuclei where the process is more advanced, the nuclear membrane is dissolved and the chromatin lies free in the cytoplasm ready for the formation of the aster. In none of these early karyokinetic figures can any abnormalities be found.

It is possible that some of the large nuclei described previously are not newly formed cells, but are the original renal cells which have withstood the attack of the toxic agent and have enlarged prior to division. This would, however, be a rather finely drawn distinction, as the large nuclei must either be cells which have but recently undergone division, or those which are about to divide.

The protoplasm surrounding these new large nuclei never shows any evidence of the carmine granules. This accords with the descriptions of Policard and of Ribbert, who both called attention to the absence of vital staining in dividing and recently divided renal cells. The contrast with those original cells which have survived the action of the toxic agent and which contain carmine granules is marked, though the scarcity of carmine in the cells of division III necessitates the study of only those specimens which are intensely stained to demonstrate this fact.

As time passes these regenerative changes become more and more

pronounced. At the end of the fifth or sixth day the large nuclei have so increased in number as to alter materially the structure of the tubule at this point. There has resulted an excessive formation of these new elements so that they now lie in masses, with little evidence of division in the surrounding cytoplasm. In this way syncytium-like masses are formed which may apparently close the lumen of the tubule (Figure 4 A). We must, however, remember the relatively small part of the tubule which is seen in one section, before drawing absolute conclusions as to the permeability of the tubule, for, as will be seen later, the permeability of the tubule is an important factor in the hydronephrotic atrophy theory.

Another appearance which is fairly common is the formation of giant-cell complexes, consisting of from six to twelve nuclei in a mass of cytoplasm which projects with a rounded contour into the lumen of the tubule (figures 4 B and 5). Their significance is the same as that of the syncytium-like masses described above, but their peculiar shape and the fact that they are described under the name of giant-cells in human chronic nephritides warrants a separate consideration. These are especially common in tubules where regeneration is most active, and for the same reason are especially common in rat kidneys. When we consider the isolation of the surviving renal cells as described in the first days of the nephritis, it seems most likely that these nuclei are the offspring of the nucleus of such an isolated renal cell, which have become heaped up as a result of the rapid proliferation.

The covering of the membrana propria proceeds slowly from the large multinuclear masses. One can see the protoplasm with the nuclei enclosed creeping along the supporting membrane (figure 5), and by this method the tubule is gradually covered with a layer of large cells which differ widely from the original epithelium. Their nuclei are still large and the chromatin stains deeply. The cytoplasm as well stains more deeply with ordinary stains than that of the normal renal cell, so that the tubules resemble superficially the collecting rather than the convoluted tubules. Another striking appearance is the excessive production of cellular elements. In many of the tubules with regeneration the epithelium consists no longer of one layer, but of irregularly, densely packed cells with

nuclei. These large oval nuclei are either placed in palisade-like formations (Thorel) or with no orderly arrangement, their long axes lying in all directions.

The normal division I of the proximal convoluted tubule with its heavy granulations of carmine undergoes relatively little change throughout the process of repair. An occasional mitotic figure is seen in it, or a group of enlarged nuclei, but there is little evidence of cellular proliferation as compared with the degenerated region of the tubule. Similarly the narrow descending limb of Henle's loop shows even less proliferation in its epithelium. The significance of these facts will be discussed in the critical review.

The effect of the detritus and casts on the regenerating cells may well be considered at this point. In the earlier stages we have seen that division III of the proximal convoluted tubules is almost entirely filled with a rather loose granular detritus, whereas the well formed hyaline casts are almost entirely limited to the collecting tubules. This condition persists throughout the first three days, and as there is little or no regeneration during this period, it is more than likely that the proliferation is at least partly retarded by the inhibitive effect of the pressure from the detritus. When regeneration does begin, however, it overcomes this resistance quite readily. The protoplasm containing the large nuclei creeps along the membrana propria beneath the detritus, and though the pressure of the latter flattens the new cells somewhat, the tubule is ultimately covered with epithelium. The shrinkage and condensation of the granular material removes much of the restraining influence and the flushing of the tubule carries the detritus into the lower levels of the kidney. As we shall see later, in considering the changes in the connective tissue, this is the most favorable outcome, for in many cases another process which leads to permanent damage to the kidney results.

Phagocytosis of the necrotic material by the regenerating epithelial cells as described by Heineke (27) has not been observed in the kidneys of any of the animals, irrespective of the toxic agent used.

The further development of the giant-cells and syncytial masses consists chiefly in an equal distribution by active growth extension of the irregularly arranged nuclei over the membrana propria (fig-

ure 5). By this means the prominence of the giant-cells is decreased and they are ultimately levelled to a uniform height, so that a single layer of cells now covers the membrana propria. In many cases, however, there is still an excess of nuclei, and these may form an irregular second layer of cells around the lumen of the tubule. Although the nuclei decrease somewhat in size and lose their vesicular appearance, they are still larger and more deeply staining than those of the normal renal cells and their protoplasm still stains deeply.

The assumption of a normal morphology by these regenerated cells in uranium nephritis is doubtless greatly hindered by the connective tissue proliferation which develops conjointly with the epithelial changes. By the tenth day, in those tubules in which regeneration occurs, there is as a rule no sign of degenerated, *i. e.*, necrotic cells, nor are there spaces of any extent yet to be covered with epithelium, yet as late as the 117th day the regenerated epithelium which is now surrounded by connective tissue still differs widely from the normal renal cells. It is, therefore, improbable that the regenerated epithelial cell in uranium nephritis ever regains the morphology of the original renal cell. As there is no sign of renal cells of abnormal morphology outside of these areas of connective tissue proliferation, the possibility cannot be denied that some of these apparently intact original cells are regenerated cells which have become indistinguishable from the normal cells around them. If one follows the process through the succeeding days, the unlikelihood of such an occurrence is evident, as the primary degeneration is not diffusely distributed, but confined to definite localized areas which correspond to the later appearing patches of connective tissue.

THE PROLIFERATIVE CHANGES IN THE CONNECTIVE TISSUE.

In the first few days of the acute nephritis the membrana propria which has been denuded of its epithelial covering remains apparently passive. It is not until the sixth or seventh day, or in severe cases, as late as the ninth, that one notices any reaction on its part.

At this time the cells of the membrana propria, which are normally few in number, small and thin, and enclosed in the fibrils of connective tissue which compose the membrane, begin to show signs

of proliferation. Instead of isolated cells they appear in groups of two or three, are twice their normal size and so project somewhat beyond the limits of the fibrous membrane into the lumen of the tubule (figure 6). The connective tissue fibrils are often separated by this process, the normally homogenous appearing membrane showing its constituent elements. These appearances are best seen in those tubules in which there is little or no regeneration, but which are filled with granular detritus.

At about the same period there occurs in such tubules a marked infiltration of the detritus with leucocytes, principally polymorphonuclears and some lymphocytes, which fill the contents of the tubule to such a degree as almost to replace the formerly light staining detritus (figures 6 and 7). At the same time in the intertubular spaces connective tissue cells may be seen in active proliferation, as is evidenced by their increase in number, large size, and fusiform shape. These cells have the typical morphology of the fibroblast, with elongated nuclei and scanty protoplasm.

Not only do these proliferating fibroblasts fill in between the tubules, but invasion of the leucocyte-infiltrated detritus occurs. The typical fusiform nuclei are seen scattered among the leucocytes. Beside fusiform nuclei, many nuclei of bizarre shape, some not unlike amitotic figures, are common, and in the later stages connective tissue fibrils are formed (figure 8).

It is evident that this growth on the part of the connective tissue, which as we have stated becomes evident on about the ninth day, can lead not only to an obliteration of the lumen by ingrowth, but to a collapse of its walls as well. The polymorphonuclear phagocytes remove the granular detritus, leaving the field open to the lymphocytes and to the fibroblasts which rapidly grow in and form connective tissue fibrils.

The effect of the obliteration of division III of the proximal convoluted tubule makes itself felt especially on that part of the renal tubule lying proximal to the occlusion. As these distension phenomena have received much attention by Aschoff and Suzuki in their theory of the pathogenesis of the uranium contracted kidney, we shall return to the early stages of the nephritis for a complete consideration of them.

In the acute stages of the nephritis, when the entire division III of the proximal convoluted tubule is packed with a dense mass of granular detritus and when the number of casts is at its maximum, there is no dilatation of the proximal convoluted tubule, nor do the capsules of the glomeruli show any distension (figure 2). This fact is easily determined in vitally stained kidneys, as here the heavily granulated tubules would be concerned. In many specimens, however, there is considerable distension of the collecting tubules, both in the medulla, medullary rays, and in the cortex, and to some extent the same appearance is seen in the distal convoluted tubule and the ascending limb of Henle's loop. The dilatation of these latter divisions may well be due to the hyaline casts in the lower levels of the collecting tubules in the medulla, as the amount of distension varies directly with the number of these casts.

A factor which must be considered in relation to the dilatation of the tubules is the variance observed in the lumen of the tubule in different functional states of the renal secretion. It is well known that there is a comparative distension of the glomeruli and the tubules throughout the kidney in active diuresis. This source of error is easily avoided, however, by giving no carmine injection immediately before the death of the animal, thus avoiding the period of renal hyperexcretion which immediately follows the injection of the vital stain.

As the process of connective tissue development proceeds, a moderate degree of distension appears, which gradually increases with the contraction of the fibrous tissue and the resulting tubular destruction. The latter, as we have seen, begins about the ninth day, and similarly at about this period beginning distension of the upper portion of the proximal convoluted tubule becomes evident. By the twenty-first day there is a marked dilatation of Bowman's capsule, with the formation of glomerular cysts, and some dilatation of the heavily granulated proximal convoluted tubules, though these last divisions never show any extreme distension (figure 9). There is, therefore, little comparable to the process of connective tissue formation that we find in hydronephrotic atrophy, as the distension in this case is an effect and not a cause of connective tissue formation.

The processes described in the epithelial regeneration and in the

connective tissue proliferation result in a striking change in the morphology of the kidney cortex. Throughout its lower layers one sees areas which stand out distinctly from the normal tubules and which give a mottled appearance to the section. In the earlier stages, when the connective tissue is just beginning to proliferate and the necrotic material is infiltrated with leucocytes, these areas are in the form of ill defined, rather widely scattered strands of deeply staining nuclei (figure 10). As the growth of connective tissue increases, these diffuse strands coalesce to form denser areas of mixed proliferating connective tissue and enlarged regenerated epithelial cells, which are sharply demarcated from the lighter staining normal tubules (figure 1). These areas persist throughout the succeeding days, until the beginning of the shrinkage of the connective tissue and the collapse of the affected tubules (fifteenth to twentieth day). By these two processes the areas are contracted to much less than their former size and by extension of the connective tissue growth around the upper divisions of the proximal convoluted tubule, glomeruli are finally included within the fibrotic patches (figure 9).

By means of this extension around the primarily unaffected proximal convoluted tubules, these areas, originally located deep in the substance of the cortex, gradually reach the cortex corticis, and by means of the connective tissue shrinkage which is progressing in them form the scars or dimples seen on the surface. Originally broad laterally, these areas now form narrow bands extending from the junction of the medulla and cortex to the capsule in ray-like processes, which become more fibrotic as time elapses.

The relation of the connective tissue proliferation to the blood vessels is a difficult one to decide. As practically all the later specimens show, the areas of connective tissue proliferation lie in proximity to the large vessels. This fact, originally pointed out by Dickson, has been explained by the opponents of the inflammatory theory as being a coincidence, as the vessels are most numerous where the epithelial damage is most marked; *i. e.,* at the junction of the cortex and medulla. This cannot be denied, nor is there any evident way to settle the question morphologically. A more detailed discussion of this problem will be given in the critical review.

CRITICAL REVIEW.

In comparing the results of the present investigations with previous work on allied subjects, we shall first consider the regeneration of the epithelial elements.

The experiments of Thorel (21) are the only ones which correspond closely with the methods employed in our work. In the regeneration following bichromate poisoning in rabbits, he describes the sudden appearance on the third day of peculiar, deeply staining nuclei in those tubules which have been cleared of detritus. Mitotic figures are common, five or six to the field, and of varying size. During the fourth and fifth day there is an extension of this process of the clearing away of detritus and the springing up of small cells. In the late period (fifth to ninth day), there is a break in the histological picture of the process due to the sudden appearance of large nuclei. These large cells are of various shape, oval, round, cuboid, or irregular, due to the pressure exerted upon them by the neighboring cells. In some places they possess separate protoplasm, while in other regions there is a syncytial formation. The nuclei measure 8 to 10 μ as contrasted with 6 μ of the normal renal nuclei, and are of an oval shape with a vesicular structure, a distinctly formed chromatin network, and one or two nucleoli.

In speaking of this sudden change and its relation to the preceding process, Thorel says: In our investigations the further course of the process of healing *unfortunately* breaks off so sharply and suddenly on the fifth day of the nephritis *that it was impossible to trace the blending of the above described processes and the transition of the individual newly lined tubules with their histologically so varying cell-lining into the harmonious regenerative processes after the fifth day of the nephritis.*[2]

The marked differences in the above description and in the processes described here for uranium poisoning are easily reconciled if we consider the small nucleated cells not as regenerated cells, as Thorel did, but as the original epithelium which has been slightly affected by the toxic agent and not entirely destroyed. In the first two days of the uranium nephritis many cells according perfectly with the small nuclei of Thorel are seen scattered throughout the damaged regions of the cortex. Not only do they closely resemble pycnotic nuclei, but, as we have shown, their reaction to the vital stain is not that of regenerated cells, but of the original epithelium. We would, therefore, class these cells not as newly regenerated cells, but as partially affected persisting renal cells. Such a conclusion simplifies the understanding of the "late stage" of Thorel very much. The large vesicular nuclei described as appearing on the fourth or fifth days are the beginning of the regeneration, not a sudden appearance of cells which have no relation to the preceding processes, a phenomenon which Thorel could not explain. The appearance of these large nuclei in bichromate nephritis and their similarity to those found in uranium nephritis are shown in figure 11.

Other writers, Arnold (34) in tuberculosis of the kidney, and Friedländer (35) in scarlet fever, have described apparently similar cells as newly formed

[2] Thorel, C., *loc. cit.* (21), p. 407. The italics are in the original.

elements, and Tilp (25) finds them commonly in his series of human renal affections. The latter is, however, somewhat doubtful of their significance, as he says: I had rather the impression that many of these distorted and deeply staining cell nuclei, as Thorel describes them, must not be altogether regarded as newly formed, but that we might have to do with nuclear degeneration besides newly formed cells.[3]

Giant-cell complexes, which have been described by many authors (Tilp, Rössle, Oertel), are commonly found in uranium nephritis, especially in rats, where the regenerative process is very pronounced. Their number seems to be directly proportional to the rapidity of the regenerative process, and this rapidity of growth is the explanation of their origin. A solitary cell, or group of cells, divides and the resulting daughter cells redivide with such rapidity that there is a heaping up of nuclei at a localized point. As we have seen, these giant-cells ultimately spread out over the membrana propria and subdivide and so disappear. A similar fate has been described for them by Tilp. The theory of Oertel that they represent an inflammatory hyperplasia receives little support from the present experiments. It is interesting to note that somewhat similar appearances are described in active regenerative changes in the liver. The knobs described by Klebs, Meder (36), and others are probably expressions of the same process that we see in the giant-cell formation in acute nephritis.

Mitotic figures are common and would seem to be the predominant method of cell division in the regenerating epithelium. Many authors have mentioned their scarcity in proportion to the great number of regenerated cells that are seen. A careful examination in such cases will show a large number of early mitotic figures, such as the early prophase with spirem formation. From the descriptions and figures of most writers, the late prophase and anaphase with spindle formation and diaster have received the most attention, while no mention is made of the early stages of the process. By the inclusion of these frequent prophases, their number more nearly approaches that expected with the rapid regenerative changes.

In none of the mitotic figures seen was there any evidence of abnormality, such as pluripolar spindles, hyper- or hypochromatic figures, or unequal distribution of the chromatin, as is described by Thorel (23). The part played by amitosis in the regenerative processes is difficult to decide. It may have significance in the rapid division resulting in giant-cell formation, as suggestive nuclear forms have been observed here, with a corresponding absence of indirect figures. Tilp makes the same suggestion, but has seen spindle formation in the giant-cells.

The seat of origin of the new cells is a point which must be considered, as Aschoff makes use of this factor in his theory of the pathogenesis of chronic uranium nephritis. In the observations of his pupil, Suzuki, the new epithelium covering the destroyed division III of the proximal convoluted tubule is described as arising primarily in the upper end of the narrow descending limb of Henle's loop, and to a lesser degree in the remnants of division III.

We have shown that even in the severe cases where the tunica propria is practically denuded there is a persistence of a few cells at some point whose nuclei

[3] Tilp, A., *loc. cit.*

are normal, although their cytoplasm, with its scanty carmine granules, may show some slight change. When one considers the small part of the tubule present in one section, it is evident that a considerable number of cells must escape the original injury and be capable of giving rise to new cells which will fill in the defect. Such was Thorel's assumption in chromium nephritis, though through the lack of a method of differentiation between original and newly formed cells, this fact then was not so easily accessible to direct observation.

In the narrow descending limb of Henle's loop we have failed to find any proliferation of practical importance. Some signs of cellular division are occasionally present, but the same can be said of the collecting tubules, where an occasional large nucleus or mitotic figure can be seen. In fact there is much more evidence of proliferation (mitotic figures, enlarged nuclei, and excess of nuclei) in the carmine-stained divisions I and II, which lie above the damaged area. Nor could the formation of the isolated giant-cells and syncytial masses be well understood if the newly formed cells which compose them had grown upwards from the descending loop of Henle. We therefore consider that the regeneration occurs directly in the surviving cells of division III of the proximal convoluted tubule.

Two methods by which gaps in the renal epithelium are filled have been described. Podwyssozki (18) laid most importance on what he termed "regeneration *per intussusceptionem.*" An increase in renal cells at some distance from the gap, assisted by an individual cell hypertrophy, results in an increased pressure, by which the persisting cells which lie at the edge of the defect are pushed into and over the break in epithelium and so close it. Other writers, notably Ribbert (19) and Loeb (37), have described an active wandering of the epithelial cells into the defect, the former in the epithelium of the kidney and the latter in the epithelium of the skin of the guinea pig. Thorel, on the other hand, lays much weight on Podwyssozki's views and observes the development of areas of vicarious regeneration at a distance from the seat of damage.

In the present investigations the mechanism of the filling in of defects is almost entirely that described by Ribbert. The cells are seen fixed in various stages of active growth extension and their protoplasmic processes can be seen extending forwards, often beneath the mass of detritus which lies upon them. As we have previously stated, there is some evidence of proliferation in the intact, vitally stained divisions I and II of the proximal convoluted tubule. This might be considered analogous to the areas of vicarious regeneration described by Thorel, but the amount of such change is insignificant in comparison with the active processes in the damaged division III.

Before discussing the changes in the connective tissue, it will be well to review briefly the normal anatomy of the renal stroma. Disse (38) has given a review of the subject, and it is from his description, and the work of Rühle (39), that the following is taken. The connective tissue of the kidney, or stroma, is composed of a network of fibrils which Mall (40) has shown to be made up of reticular tissue and collagen fibrils. The fibrils arise directly from the adventitia of the larger vessels and surround the tubules, nerves, veins, and lymphatics. All the ramifications of the vessels are enclosed in these meshes, and, as Rühle has shown, the fibrils form the walls of the capillaries and smallest vessels, on which the endothelium lies directly. The border of fibrils which abuts the epithelium

of the tubules is somewhat condensed and forms the membrana propria. This structure has been studied by Rühle (39), who shows that the above mentioned fibrils of the stroma wind around the tubules in spirals, taking part in formation of the membrana propria, and then return, uniting with the perivascular connective tissue. Among the fibrils lie the spindle-shaped connective tissue cells, which in young animals send processes, which unite with, or constitute, the fibrils. The membrana propria, which appears in ordinary sections as a homogeneous membrane, is, therefore, only the condensed connective tissue of the kidney on which the epithelial cells lie. This, and the fact that connective tissue fibrils not only arise from the walls of the larger vessels, but even form the walls of smaller ones, are considerations whose importance in the pathological changes to be discussed cannot be overrated.

The importance of the vascular connective tissue stroma in the process of repair in the kidney has been recognized by many writers. Ziegler (41) states that an intracanalicular regeneration, as opposed to the regeneration of new tubules, is only possible when the vessel-bearing stroma is intact. If this latter is damaged there is either no regeneration, or only an abnormal epithelium is formed.

Aschoff (42) in speaking of reparative changes in the tubules says: If the tunica propria is destroyed under the influence of the inflammation, a rebuilding of the tubule in this location does not take place. Tilp (25) distinguishes two types of regenerative processes: that in which the vascular connective tissue apparatus (*"Blutgefässbindegewebsapparat"*) is not disturbed and in which there may be a complete return to normal morphology and function; and a type in which the stroma also is damaged and where repair is never complete. This latter type he describes in chronic Bright's disease, renal infarcts, tuberculosis, and tumors of the kidney.

We have seen from the description of the development of chronic uranium nephritis that there never results a morphological restitution of normal structure in the kidney, and that this lack of restitution is due to the development of connective tissue in the affected areas, which begins early and progresses steadily until the death of the animal. Two possibilities present themselves for the explanation of this fact. The connective tissue overgrowth may be due to a disturbance in the structure of the vascular connective tissue stroma, or it may be the result of a primary damage solely to the epithelial elements, with a passive ingrowth of connective tissue to fill in the dead space resulting from a collapse of the tubules. As we have stated in the introduction, the former is the view of Dickson, and is supported not only by his morphological studies, but also by the physiological investigations of those who have studied the function of uranium kidneys. A further morphological support to this theory has been given by the work of Baehr (43), who has shown that severe damage may be done the vascular apparatus, if the uranium reaches the kidney in high enough concentration. By injecting directly into the renal artery of rabbits he has produced necrosis and hemorrhage from the capillaries of the glomeruli. Chromium and other toxic substances did not produce any such condition, showing definitely that there is some specific action on the blood vessels in uranium poisoning.

The opposite view, of primary epithelial damage, has been upheld by Aschoff and Suzuki in a modified form, in which they liken the process to that observed

in hydronephrotic atrophy of the kidney. They claim that the insignificant regeneration in division III of the proximal convoluted tubule, due to the inhibitive effect of the necrotic masses and the destruction of the point from which regeneration occurs (upper part of the descending limb of Henle's loop), leads to a collapse of the tubule with resulting inactivity atrophy of the tubule proximal to this point, and resulting development of connective tissue around the atrophic tubule. They therefore characterize the kidney in chronic uranium nephritis as a "*tubuläre Schrumpfniere,*" which has no homologue in the human nephritides, with the possible exceptions of pyelonephritic contracted kidneys, uric acid kidneys, and sclerotic foci proximal to renal cysts.

The hydronephrotic atrophy theory will first be considered. That part of the theory which has to do with the regenerative changes in the damaged tubule and the origin of the new cells has already been dealt with. A special study was made of appearances which might be interpreted as a hydronephrotic change, and as evidence of such a process the dilatation of the tubule above the point of obstruction was taken. It has been seen that dilatation of the proximal convoluted tubule and of the glomerular space is lacking until the twelfth day, a period considerably later than the beginning of connective tissue proliferation. In the earlier stages, although there is a marked filling of the tubule with detritus and casts, there is practically no distension. As the process proceeds and the connective tissue becomes more fully developed, the dilatation of the proximal convoluted tubule and glomerular capsule occurs and reaches its maximum about the twenty-fifth day, when marked connective tissue shrinkage is present. The collecting tubules show a dilatation from the first, due to the presence of many hyaline casts in the lower levels of the medulla, but there is never any evidence of connective tissue proliferation around them. To recapitulate, in the acute stages of epithelial degeneration, there is no sign of dilatation in the tubule above the seat of lesion, while in the later stages dilatation begins with the development of the connective tissue, and increases in direct proportion to it.

An especially good demonstration of the fact that the late dilatation phenomena in divisions I and II of the proximal convoluted tubules are due to connective tissue shrinkage is found in kidneys of animals suffering from a spontaneous chronic nephritis at the time of the acute attack from the uranium injection. In those areas where there is no spontaneous chronic process, division III of the proximal convoluted tubules shows the typical acute necrosis and its lumen is filled with detritus, yet there is no dilatation in the corresponding divisions I and II of the glomerular spaces above. In the areas of preëxisting chronic nephritis where there is marked proliferation of connective tissue and resulting collapse of the tubules, there is marked dilatation of the upper divisions of the proximal convoluted tubules and cyst formation in the glomerular spaces.

These facts cannot be reconciled with the hydronephrotic theory, but point directly to primary vascular connective tissue irritation with resulting proliferation. The same epithelial damage in division III of the proximal convoluted tubule is seen following the use of other toxic agents. In fact, in sublimate poisoning the epithelial degeneration is more severe than that which follows uranium administration, and the epithelial detritus is much more persistent and is often ultimately calcified, yet there is no tendency to the formation of a progressive chronic nephritis.

We therefore believe that a purely parenchymatous theory, one based solely on the reaction of the epithelial elements to the toxic agent, fails to explain the pathogenesis of the chronic uranium nephritis. The connective tissue reaction is too early and active to be considered a purely secondary filling in of dead space, nor can it be explained by a hydronephrotic theory. We therefore consider that the proliferation of the connective tissue in chronic uranium nephritis is due to a primary direct stimulation by the uranium, which so differs in this regard from the other renal toxic agents.

CONCLUSIONS.

1. Proliferation of connective tissue in chronic uranium nephritis is early and progressive and due to a direct stimulation on the part of the uranium.

2. The hydronephrotic theory fails to explain the phenomena observed in the progress of the chronic nephritis.

3. Carmine is deposited, as shown by Suzuki, in the proximal convoluted tubule only, and in different amounts in different levels of this segment of the tubule, so that subdivisions may be made.

4. Regeneration begins about the fourth day, but varies in proportion to the dose of renal irritant given.

5. The first cells to appear are large vesicular elements, and there is a gradual development of syncytium-like masses and giant-cells. These last are due to the rapidity of cell division.

6. Small, deeply staining nuclei, which are morphologically identical with the newly regenerated cells described by Thorel and others, are present from the first day of the acute nephritis and are persisting renal cells which have been slightly affected by the toxic agent.

In conclusion I wish to thank Dr. E. C. Dickson and Dr. W. Ophüls for their guidance and interest, and for their frequent aid in the progress of this work.

BIBLIOGRAPHY.

1. Dickson, E. C., A Report on the Experimental Production of Chronic Nephritis in Animals by the Use of Uranium Nitrate, *Arch. Int. Med.*, 1909, iii, 375; A Further Report on the Production of Experimental Chronic Nephritis in Animals by the Administration of Uranium Nitrate, *ibid.*, 1912, ix, 557.

2. Christian, H. A., A Glomerular Lesion of Experimental Nephritis, *Boston Med. and Surg. Jour.*, 1908, clix, 8.

3. Suzuki, T., Zur Morphologie der Nierensekretion unter physiologischen und pathologischen Bedingungen, Jena, 1912.

4. Folin, O., Karsner, H. T., and Denis, W., Nitrogen Retention in the Blood in Experimental Acute Nephritis of the Cat, *Jour. Exper. Med.*, 1912, xvi, 789.
5. MacNider, W. deB., A Study of the Renal Epithelium in Various Types of Acute Experimental Nephritis and of the Relation Which Exists between the Epithelial Changes and the Total Output of Urine, *Jour. Med. Research*, 1912, xxvi, 79.
6. Christian, H. A., Experimental Nephritis, *Boston Med. and Surg. Jour.*, 1908, clviii, 416, 452.
7. Richter, P. F., Experimentelles über die Nierenwassersucht, *Berl. klin. Wchnschr.*, 1905, xlii, 384.
8. Schlayer and Hedinger, Experimentelle Studien über toxische Nephritis, *Deutsch. Arch. f. klin. Med.*, 1907, xc, 1.
9. Georgopulos, Experimentelle Beiträge zur Frage der Nierenwassersucht, *Ztschr. f. klin. Med.*, 1906, lx, 411.
10. Pearce, R. M., The Production of Edema; An Experimental Study of the Relative Etiologic Importance of Renal Injury, Vascular Injury and Plethoric Hydremia, *Arch. Int. Med.*, 1909, iii, 422.
11. Ophüls, W., Experimental Nephritis in Guinea-Pigs by Subcutaneous Injections of Chromates, *Proc. Soc. Exper. Biol. and Med.*, 1911–12, ix, 11.
12. Kabierske, E., Die Chromniere, Dissertation, Breslau, 1880.
13. Pander, H., Beiträge zur Chromwirkung, Dissertation, Dorpat, 1887.
14. Smith, R. M., Experimental Chronic Nephritis, *Arch. Int. Med.*, 1911, viii, 481.
15. Ophüls, W., Experimental Chronic Nephritis, *Jour. Am. Med. Assn.*, 1907, xlviii, 483.
16. Aschoff, L., Zur Morphologie der Nierensekretion unter physiologischen und pathologischen Bedingungen, *Verhandl. d. deutsch. path. Gesellsch.*, 1912, xv, 199.
17. Peter, K., Untersuchungen über Bau und Entwicklung der Nieren, Jena, 1909.
18. Podwyssozki, W., Jr., Ueber die Regeneration der Epithelien der Leber, der Niere, der Speichel- und Meibom'schen Drüsen unter pathologischen Bedingungen, *Fortschr. d. Med.*, 1885, iii, 630; Die Gesetze der Regeneration der Drüsen Epithelien unter physiologischen und pathologischen Bedingungen, *ibid.*, 1887, v, 433.
19. Ribbert, H., Zur Regeneration der Leber und Niere, *Arch. f. Entwcklngsmechn. d. Organ.*, 1904, xviii, 267.
20. Hochhaus, H., Ueber Gewebsveränderungen nach localer Kälteeinwirkung, *Virchows Arch. f. path. Anat.*, 1898, cliv, 320.
21. Thorel, C., Pathologisch-anatomische Beobachtungen über Heilungsvorgänge bei Nephritis. Eine experimentelle und kritische Studie, *Deutsch. Arch. f. klin. Med.*, 1903, lxxvii, 29, 395, 470.
22. Cornil and Toupet, Sur la karyokinèse des cellules épithéliales et de l'endothélium vasculaire observée dans le rein à la suite de l'empoisonnement par la cantharidine, *Arch. de physiol. norm. et path., Series 3*, 1887, x, 71.
23. Thorel, C., Über Mitosen und atypische Regenerationen bei Nephritis, *Deutsch. Arch. f. klin. Med.*, 1905, lxxxiv, 173.
24. Rössle, R., Störungen der Regeneration von Nierenepithelien, *Virchows Arch. f. path. Anat.*, 1902, clxx, 375.
25. Tilp, A., Ueber die Regenerationsvorgänge in den Nieren des Menschen, Jena, 1912.

26. Oertel, H., On Epithelial Proliferation and the Formation of Epithelial Giant-Cells in Nephritis, *Publications of the Russell Sage Institute of Pathology*, 1909, No. 1.
27. Heineke, A., Die Veränderungen der menschlichen Niere nach Sublimatvergiftung mit besonderer Berücksichtigung der Regeneration des Epithels, *Beitr. z. path. Anat. u. z. allg. Path.*, 1909, xlv, 197.
28. Policard, A., Le tube urinaire des mammifères, *Rev. gén. d'histol.*, 1908, iii, 307.
29. Chrzonszczewsky, N., Zur Anatomie der Niere, *Virchows Arch. f. path. Anat.*, 1864, xxxi, 153.
30. Ribbert, H., Die normale und pathologische Physiologie und Anatomie der Niere, *Bibliot. med., Abt. C*, 1896, No. 4.
31. Ribbert, H., Die Abscheidung intravenös injizierten gelösten Karmins in den Geweben, *Ztschr. f. allg. Physiol.*, 1904, iv, 201.
32. Ophüls, W., Occurrence of Spontaneous Lesions in Kidneys and Livers of Rabbits and Guinea Pigs, *Proc. Soc. Exper. Biol. and Med.*, 1910–11, viii, 75.
33. Christian, H. A., and O'Hare, J. P., Glomerular Lesions in Acute Experimental (Uranium) Nephritis in the Rabbit, *Jour. Med. Research*, 1913–14, xxviii, 227.
34. Arnold, J., Beiträge zur Anatomie des miliaren Tuberkels. II. Ueber Nierentuberculose, *Virchows Arch. f. path. Anat.*, 1881, lxxxiii, 289.
35. Friedländer, C., Ueber Nephritis scarlatinosa, *Fortschr. d. Med.*, 1883, i, 81.
36. Meder, E., Ueber acute Leberatrophie, mit besonderer Berücksichtigung der dabei beobachteten Regenerationserscheinungen, *Beitr. z. path. Anat. u. z. allg. Path.*, 1895, xvii, 143.
37. Loeb, L., Über Regeneration des Epithels, *Arch. f. Entwcklngsmechn. d. Organ.*, 1898, vi, 297.
38. Disse, J., Harnorgane, *Handb. d. Anat. d. Mensch.*, 1902, vii, 87.
39. Rühle, G., Ueber die Membrana propria der Harnkanälchen und ihre Beziehung zu dem interstitiellen Gewebe der Niere, *Arch. f. Anat. u. Physiol., Anat. Abt.*, 1897, 153.
40. Mall, F., Das retikulierte Gewebe und seine Beziehungen zu den Bindegewebsfibrillen, *Abhandl. d. k. sächs. Gesellsch. d. Wissensch., math.-phys. Cl.*, 1891, xvii, 295.
41. Ziegler, E., Lehrbuch der allgemeinen Pathologie und der pathologischen Anatomie, 11th edition, Jena, 1906, ii, 838.
42. Aschoff, L., Pathologische Anatomie. Ein Lehrbuch für Studierende und Aerzte, Jena, 1909, ii, 488.
43. Baehr, G., Über experimentelle Glomerulonephritis (ein Beitrag zur Lehre der Schrumpfniere), *Beitr. z. path. Anat. u. z. allg. Path.*, 1913, lv, 545.

EXPLANATION OF PLATES.

Plate 23.

Fig. 1. Guinea pig 51. Uranium; killed on fifteenth day. The same region as the section in figure 10. The diffusely scattered strands of the earlier stage have coalesced to form a dense area of large regenerated epithelial cells and proliferated connective tissue which fills the lower half of the figure. A glomerulus

lies adjacent to this area and is being surrounded by the process, the effect of this being shown by the beginning dilatation of the capsular space. The divisions of the proximal convoluted tubules and collecting tubules at the upper margin of the figure are entirely normal in structure, though somewhat dilated. Spencer obj. 16 mm. Compensating ocular 9 ×.

PLATE 24.

FIG. 2. Rat 9. Uranium; killed on sixth day. The entire cortex and the outer stripe of the outer zone of the medulla are shown. In the upper two-thirds of the figure are seen the normal divisions I of the proximal convoluted tubule filled with carmine granules in rod-like formations, which show as an intense black in the photograph, and which obscure all details in the structure of these tubules (Div. I). A small area of round cell infiltration is seen at A, and below this is a group of regenerated tubules with deeply staining large nuclei and dense protoplasm (Reg. Div. III). To the left of this group is a glomerulus, which separates the cortex and the outer stripe of the outer zone of the medulla, with its heavily granulated division I, and below are seen many tubules whose epithelium is entirely destroyed and whose lumen is filled with homogeneous detritus (Div. III). Spencer obj. 16 mm. Compensating ocular 9 ×.

PLATE 25.

FIG. 3. Rat 7. Bichromate; killed on fifth day. Similar view of cortex and medulla as is in figure 2. In the upper quarter of the section surrounding the intact glomeruli are seen the divisions I of the proximal convoluted tubules with their epithelium destroyed and their lumen filled with detritus (Div. I). In the medullary ray which extends diagonally across the middle of the figure are seen sections of the thick ascending limb of the loop of Henle with pycnosis of the nuclei and desquamation of the epithelium. In the outer stripe of the outer zone of the medulla are seen the well preserved divisions III which appear darker in the photograph, due to the presence of the carmine granules which they contain (Div. III). Spencer obj. 16 mm. Compensating ocular 9 ×.

PLATE 26.

FIG. 4. Rat 9. Uranium; killed on fifth day. The figure is taken from the area of severest degeneration, *i. e.*, the outer stripe of the outer zone of the medulla, where marked regenerative changes are clearly seen. At A is a tubule whose lumen is almost entirely filled with a syncytium-like mass of protoplasm with large vesicular nuclei. Similar large deeply staining nuclei are seen in many of the tubules. At B is a typical giant-cell with its rounded projection into the lumen of the tubule. Spencer obj. 16 mm. Compensating ocular 9 ×.

FIG. 5. The tubule B of figure 4 is shown. At the lower end of the section of the tubule is seen a rounded mass of protoplasm which contains six enlarged deeply staining nuclei. On both sides of this giant-cell are seen large nuclei with their surrounding protoplasm which, by growth extension, are creeping along the bare membrana propria. Above the giant-cell is a degenerated cell, out of focus, which obscures the lumen at this point. Spencer obj. 4 mm. Compensating ocular 9 ×.

PLATE 27.

FIG. 6. Detail of figure 7. The tubule shown is the one in the center of figure 7, lying between the long horizontal degenerated tubule which is filled with detritus, and the oval-shaped collecting tubule. The infiltrating leucocytes are seen to be composed of both polymorphonuclear leucocytes and lymphocytes. At the lower edge of the infiltrated tubule is seen a densely staining row of fusiform proliferating tunica propria cells. Spencer obj. 4 mm. Compensating ocular 9 ×.

FIG. 7. Guinea pig 4. Uranium; killed on ninth day. Across the middle of the figure stretches a row of tubules whose epithelium is entirely destroyed and whose lumen is filled with light staining granular detritus. Scattered throughout the sections are tubules whose lumens are filled with leucocytes, and in other places these leucocytic infiltrations occur between the tubules. There is also marked proliferation of the tunica propria cells, which aid in the formation of the densely staining areas of cellular accumulation. Spencer obj. 16 mm. Compensating ocular 9 ×.

PLATE 28.

FIG. 8. Guinea pig 4. Uranium; killed on ninth day. In the center of the section is a large tubule filled with light staining detritus. Extending upwards from the lower end of the section of the tubule is a strand of fusiform fibroblasts, which scatter out irregularly throughout the detritus. In one place beginning fibril formation by these cells is seen. On the right is a deeply staining strand of proliferating tunica propria cells and a glomerulus. Spencer obj. 4 mm. Compensating ocular 9 ×.

PLATE 29.

FIG. 9. Guinea pig 40. Uranium; killed on twenty-first day. Same region as figures 1 and 10. The glomeruli are completely enclosed in the mass of proliferating fibrous tissue and atypical regenerated epithelial cells, and there is marked cystic formation in their capsular spaces. The shrinkage of the connective tissue has lessened the area of changed kidney tissue considerably, and these rays of fibrous tissue now extend to the capsule. The collecting tubules on each side of this area are normal in structure and somewhat dilated. Spencer obj. 16 mm. Compensating ocular 9 ×.

FIG. 10. Guinea pig 42. Uranium; killed on ninth day. The figure is taken from the lower layer of the cortex adjacent to the medulla. Throughout the section are dark, intensely staining strands which consist of proliferated tunica propria cells around the collapsed tubules. The contrast of the diffuse proliferation of the connective tissue as contrasted with the later stages is well shown. Casts are seen in the collecting tubules, and there is some dilatation of these tubules. The glomeruli are normal. Spencer obj. 16 mm. Compensating ocular 9 ×.

FIG. 11. Rat 7. Bichromate; killed on fifth day. In the center of the figure is a group of tubules with deeply staining large nuclei and rather dense protoplasm. In places there is an excessive number of nuclei and they are seen heaped upon each other in irregular layers. The tubules surrounding these regenerated segments are practically normal. Spencer obj. 16 mm. Compensating ocular 9 ×.

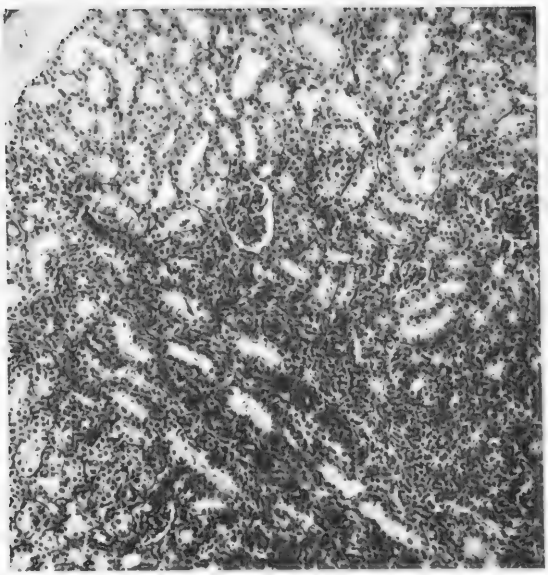


FIG. 1.

(Oliver: Histogenesis of Chronic Uranium Nephritis.)

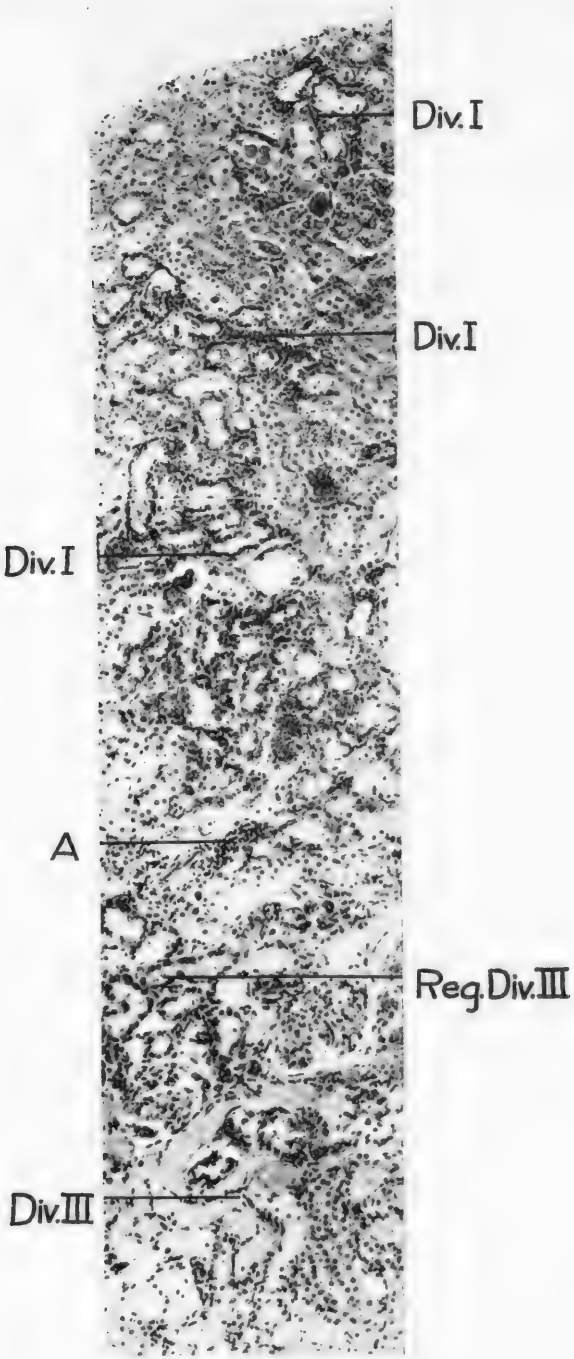


FIG. 2.

(Oliver: Histogenesis of Chronic Uranium Nephritis.)

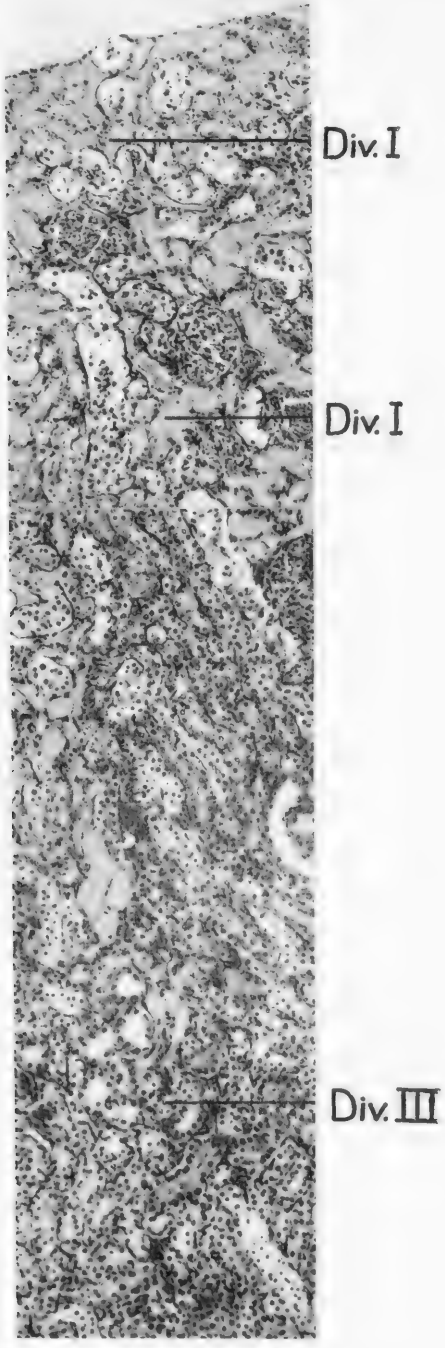


FIG. 3

(Oliver: Histogenesis of Chronic Uranium Nephritis.)

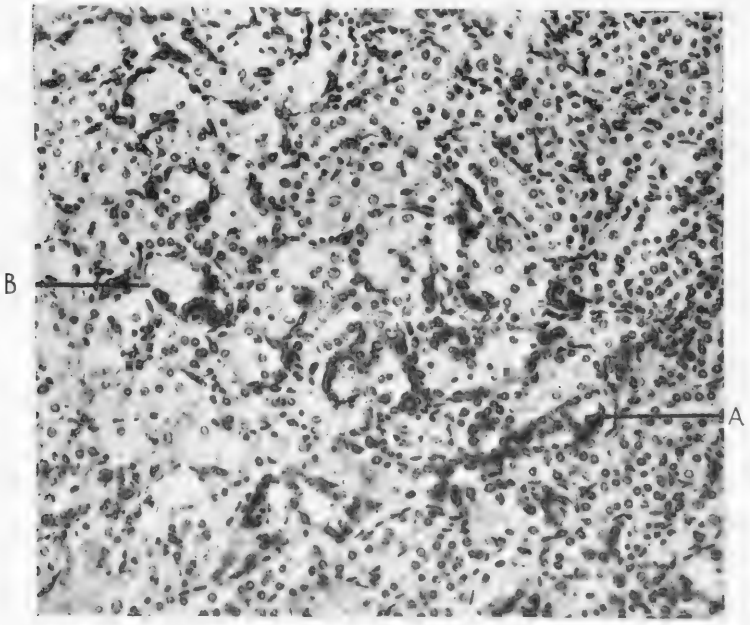


FIG. 4.

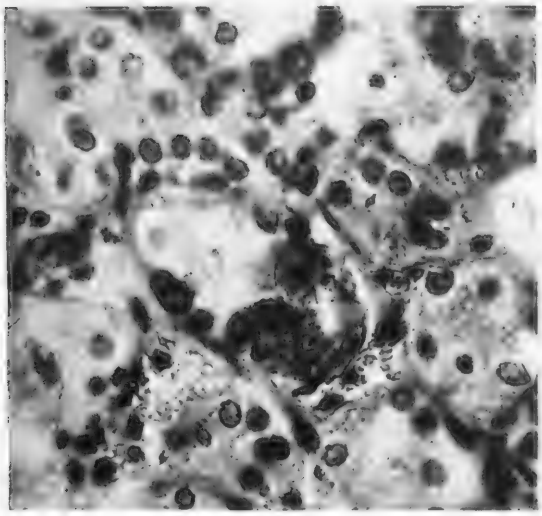


FIG. 5.

(Oliver: Histogenesis of Chronic Uranium Nephritis.)

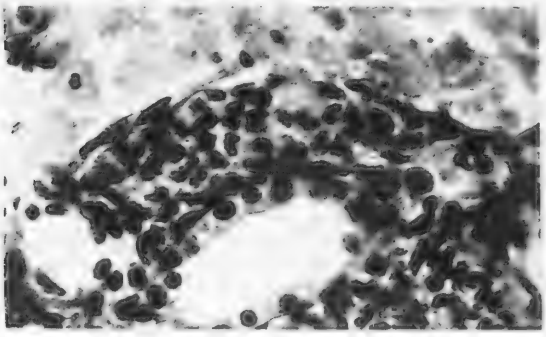


FIG. 6.

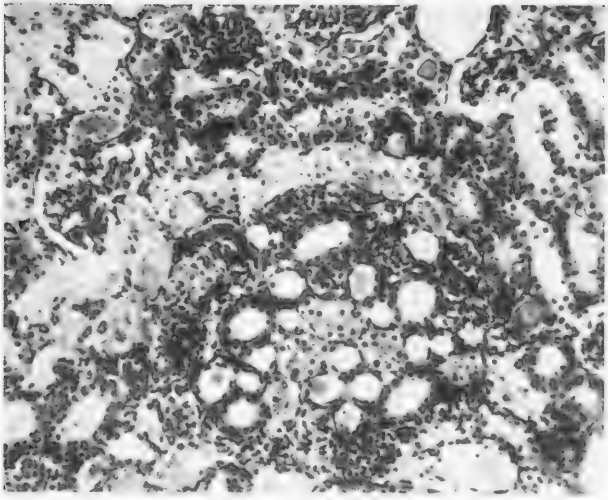


FIG. 7.

(Oliver: Histogenesis of Chronic Uranium Nephritis.)

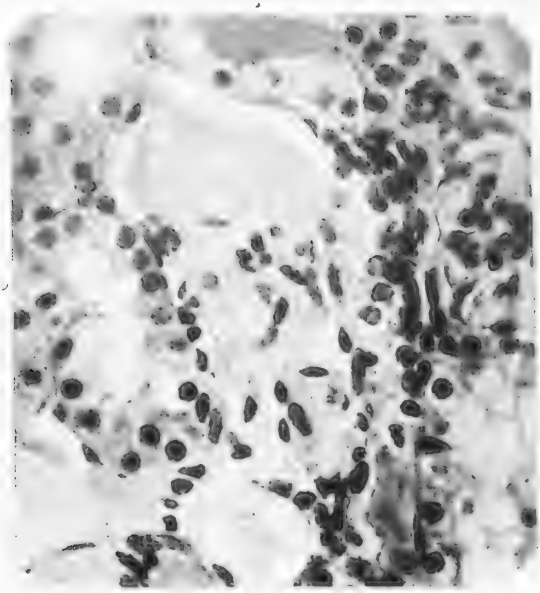


FIG. 8.

(Oliver: Histogenesis of Chronic Uranium Nephritis.)

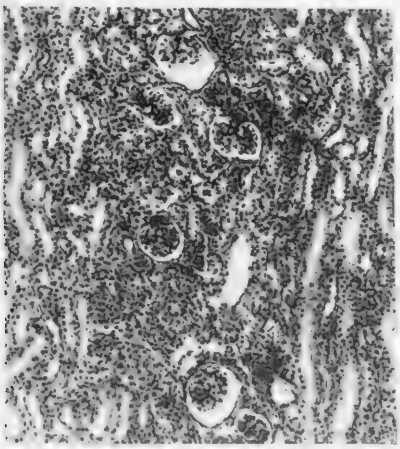


FIG. 9.

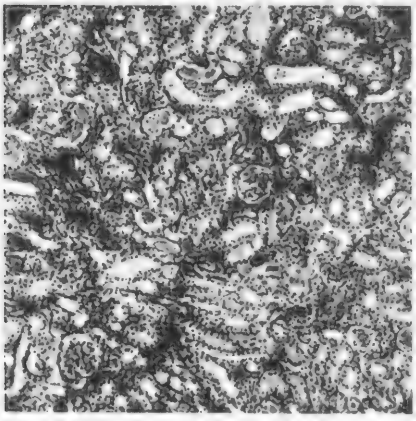


FIG. 10.

FIG. 11.

(Oliver: Histogenesis of Chronic Uranium Nephritis.)

FIG. 12.

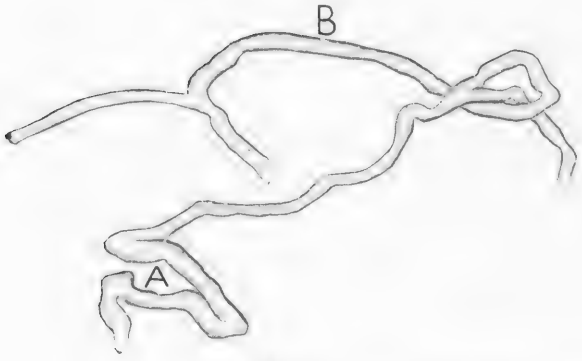


FIG. 13.

(Oliver: Histogenesis of Chronic Uranium Nephritis.)

PLATE 30.

FIG. 12. Normal vitally stained rabbit (trypan blue). The proximal convoluted tubule with the upper part of its medullary portion is shown. That part of the tubule lying adjacent to the glomerulus is heavily impregnated with vitally stained granules, so that the unstained nuclei stand out in contrast as lighter areas. The number of granules decreases as one departs from the glomerulus, appearing as isolated groups and finally disappearing completely. Camera lucida drawing of isolated tubule. Bausch and Lomb obj. 1. Ocular 2/3.

FIG. 13. Normal vitally stained rabbit. The second convoluted tubule is seen at (A), connecting with the collecting tubule (B). The second convoluted tubule, as shown in Peter's illustrations, consists of but few loops as compared with the proximal convoluted tubule, and contains no vitally stained granules. Camera lucida drawing of isolated tubule. Bausch and Lomb obj.1. Ocular 2/3.

OBSERVATIONS ON THE ETIOLOGY OF GOITRE IN BROOK TROUT.

IV. The Effect of Feeding with Fresh and Stale Liver.*

By DAVID MARINE, M.D.

(From the H. K. Cushing Laboratory of Experimental Medicine of Western Reserve University, Cleveland.)

This report includes (1) a brief summary of the sixth annual inventory of the state of the thyroid glands in the brook trout at the hatchery of the Blooming Grove Hunting and Fishing Club, and (2) an account of some experiments in the feeding of fresh and stale liver.

The arrangement and number of the ponds and troughs, the water supply, the strain of brook trout, and their distribution in the ponds and troughs according to age, have remained unchanged since our first observations in 1909. The crowding has gradually increased during these years, but the general external appearance of cleanliness of the ponds and troughs has not changed. Those containing the fry have always been unusually well cared for. The food, consisting of hog's liver and heart, has remained constant during the six years for all fish up to the ninth month of life, while for the past three years all fish over nine months old have been fed with hashed sea fish.

Histological examinations of the thyroids of a complete series representing specimens from all the ponds and troughs have been made yearly. The condition of the thyroid up to the time the change of food is made, *i. e.*, the ninth month of life, has not varied noticeably during these six years. All have shown marked active thyroid overgrowth, as noted in previous papers. When the food is changed to sea fish at the ninth month, the thyroid overgrowth is arrested, and the gland returns to its colloid or resting stage in about thirty-five to forty days (1). During the three years that this effect has been studied, no further hypertrophy or growth of the thyroid has been observed, although the fish remain in the ponds for a period of about two years after the change of food is instituted. On the other hand, when liver was used as the food throughout their lives in captivity, the thyroid overgrowth progressed continuously to visible external manifestations in practically all the fish by the end of the second year. The substitution of sea fish as a food has proved to be a specific curative and preventive measure under apparently the same conditions where liver as food caused continuous thyroid overgrowth.

* Received for publication, February 24, 1915.

In previous papers (2, 3, 4) it has been suggested that the volume of water, its oxygen supply, its content of excreta (overcrowding), and the highly artificial food (liver and heart muscle) might be factors in causing the thyroid overgrowth. It was on account of the rapid regression of the thyroid overgrowth when sea fish was fed, and on account of its continuous growth when liver and heart muscle were fed, that food was more particularly suspected of playing an important part in stimulating the thyroid to this continuous growth. If this was a factor, it seemed probable that simple experiments in which the freshest liver was fed and experiments in which distinctly stale liver was fed might reveal differences in the thyroid growth. To this end the following experiments were made.

Two troughs were selected from the series of twenty-one. The fry of one were fed in the usual manner (twice daily) with the freshest liver, while the fry of the others were fed with portions of the same hashed liver as above, which had been kept in the cold chamber (11° to 15° C.) for two days. The experiments were begun on July 16 and terminated September 17, at which time the food of all fry was changed to sea fish. Two fish were taken at weekly intervals from each trough. As controls, specimens were taken from the twenty-one troughs at the beginning of the experiment, and two specimens at weekly intervals from one adjoining trough, which were fed on the general stockroom supply. At the end of the two months there were no gross changes in size, activity, or general appearance in the two sets. Histological preparations were made from all these thyroids. Study of the condition of the thyroids showed that there was a slight gradual increase in the degree of thyroid overgrowth noticeable in the second month in those fed with the freshest liver over those fed with the same liver held for two days. No difference could be distinguished between those fed with the freshest liver and the controls fed from the general supply.[1]

[1] Similar experiments have been carried out on rats, where better control could be had. Thus two series of twenty-one young rats each were divided into groups of three each. The first group was fed on the fresh hog's liver, from animals killed the same day; the second group was fed with the same amount of the same liver one day old; and each subsequent group was fed with the same liver one day older than the preceding group. It was kept at room temperature screened from flies. They were fed with liver six times weekly, while bread and water were kept continuously in the cages. All gained in weight, those getting the freshest liver slightly more than those getting the staler liver. One rat in each group was killed at intervals of two weeks, and the thyroids were examined microscopically. There was distinct hypertrophy, as judged by the reduction in stainable colloid and increase in the size of thyroid cells, in those groups getting liver 1, 2, and 3 days old, while those getting fresh liver and liver 4, 5, and 6 days old had normal or nearly normal glands.

These findings were the reverse of what I thought might occur, as I had in mind the possibility that autolysis and bacterial digestion of the liver might produce substances capable of stimulating the thyroid cells to increased activity.

A plausible explanation for these findings is not at hand. The lack of control over the amounts of liver taken by the fish seemed suggestive, but experiments with rats where the quantity was controlled show in general the same results. The idea that certain products of autolysis and bacterial decomposition of the liver act as irritants to the thyroid may be abandoned. Since controlled experiments show only a very slight thyroid hypertrophy, one may conclude that the diet is only a contributing factor, and that it may act by increasing the work of the thyroid in order to maintain a general increase in metabolism, especially in connection with the overfeeding of a nutritionally incomplete diet.

BIBLIOGRAPHY.

1. Marine, D., The Rapidity of the Involution of Active Thyroid Hyperplasias of Brook Trout Following the Use of Fresh Sea Fish as a Food, *Jour. Exper. Med.*, 1914, xix, 376.
2. Marine, D., and Lenhart, C. H., Observations and Experiments on the So-Called Thyroid Carcinoma of Brook Trout (*Salvelinus fontinalis*) and Its Relation to Ordinary Goitre, *Jour. Exper. Med.*, 1910, xii, 311.
3. Marine and Lenhart, Further Observations and Experiments on the So-Called Thyroid Carcinoma of the Brook Trout (*Salvelinus fontinalis*) and Its Relation to Endemic Goitre, *ibid.*, 1911, xiii, 455.
4. Marine, Further Observations and Experiments on Goitre (So Called Thyroid Carcinoma) in Brook Trout (*Salvelinus fontinalis*). III. Its Prevention and Cure, *ibid.*, 1914, xix, 70.

THE ORIGIN AND STRUCTURE OF A FIBROUS TISSUE WHICH APPEARS IN LIVING CULTURES OF ADULT FROG TISSUES.*

By GEORGE A. BAITSELL, Ph.D.

(*From the Osborn Zoölogical Laboratory of Yale University, New Haven.*)

Plates 31 to 36.

INTRODUCTION.

Within the past few years a considerable amount of investigation has been done upon the cultivation of living tissues outside the body, and various phases of the subject have been studied. Thus far, however, it appears that there has been very little consideration of the reactions that occur in the plasma clot of the tissue cultures. Harrison,[1] among others, has shown that a supporting framework of some sort is necessary in the medium in which the living tissue is imbedded in order to obtain a prolific growth and movement of the tissue cells. The fibrin net which is present in plasma or lymph cultures forms this framework. If the plasma or lymph be defibrinated and the resulting serum used as a medium in which to cultivate the tissues there is, in general, comparatively little growth or movement of the tissue cells. In this paper[2] the results of experiments with living cultures of adult frog tissues are presented, which show that a definite reaction frequently occurs in the plasma clot. As a result the fibrin net present in the clot gradually loses its typical structure and becomes transformed into a new fibrous tissue closely resembling in its appearance, structure, and function various types of white fibrous connective tissues.

The thanks of the author are due to Professor Ross G. Harrison

* Received for publication, March 4, 1915.

[1] Harrison, R. G., The Reaction of Embryonic Cells to Solid Structures, *Jour. Exper. Zoöl.*, 1914, xvii, 521.

[2] A report of this work was given at the meeting of the American Society of Zoölogists held at Philadelphia, December 31, 1914.

for his suggestion of the problem and for his invaluable assistance and advice from time to time as the work progressed.

MATERIAL AND METHODS.

In the work reported in this paper, the tissue cultures have all been made by the hanging drop method, and the usual precautions to keep the cultures sterile have been observed. This method of tissue culture has become quite well known within the last few years, and a full description of the technique will not be given in this paper.[3] The plasma used has been secured in the following manner: An anesthetized frog is opened ventrally and the heart exposed. A hypodermic syringe (a five cubic centimeter glass Luer with a short fine needle is very satisfactory) is then inserted in the ventricle. If the frog has not been anesthetized too heavily, a considerable amount of blood (one to two cubic centimeters) can then be drawn out of the ventricle. When all the blood that can readily be secured —it may take some minutes—is obtained, the syringe is withdrawn and the blood centrifuged. In the present experiments it has been the rule to use only fresh plasma. In most cases both the plasma and the tissue for imbedding in it were obtained from the same animal. The tissue taken from the animal was placed in Ringer's solution and cut into small pieces.

During the course of the experiments it was found necessary to preserve and section a great many of the preparations, and in order to do this it was necessary to get the plasma clot with the imbedded tissue free from the cover-glass without injury. To accomplish this it was found convenient to coat the cover-glasses before being used with a thin coat of celloidin. This was done by immersing the cover-glasses in a weak solution and then letting them dry. The celloidin solution is sterile but if, for any reason, there is need of

[3] The reader is referred for further details regarding technique and for a general survey of the field to papers by the following authors: Harrison, R. G., The Outgrowth of the Nerve Fiber as a Mode of Protoplasmic Growth, *Jour. Exper. Zoöl.*, 1910, ix, 787. Carrel, A., and Burrows, M. T.,Cultivation of Tissues *in Vitro* and Its Technique, *Jour. Exper. Med.*, 1911, xiii, 387. Oppel, A., Causal-morphologische Zellenstudien. V. Mitteilung. Die aktive Epithelbewegung, ein Faktor beim Gestaltungs- und Erhaltungsgeschehen, *Arch. f. Entwcklngsmechn. d. Organ.*, 1912, xxxv, 371.

sterilizing the cover-glasses it can be done in a dry heat. The celloidin will stand a temperature of 100° C. without apparent change. Using these celloidin-coated cover-glasses it becomes a comparatively simple matter to get the plasma clots with the imbedded tissue free from the cover-glass. In general the clot will remain attached to the cover-glass in the killing fluid and through the washing and dehydrating up to the absolute alcohol, at which point the celloidin will be dissolved and the preparation will, in most cases, free itself from the cover-glass with no injury whatsoever. The preparation can then be imbedded and sectioned in the usual manner. Sections of the preserved preparations were cut at eight microns. Various methods of staining the preserved cultures have been tried and a description of these is given in a later section of this paper.

STUDY OF THE LIVING CULTURES.

As shown in the accompanying table (table I) a total of 996 cultures have been made from various tissues of the adult frog during the course of these experiments, and of this number 846 have been under observation and form the basis for the results reported in this paper. It is possible by a study of the living preparations to follow closely the reactions that occur in the plasma clot, and photographs

TABLE I.

Summary of the Results of the Experiments.

Material imbedded.	Total No. of cultures made.	No. of cultures rejected.	No. of cultures studied.	No. of cultures in which fibers appeared.
Liver	476	81	395	86
Spleen	170	50	120	55
Muscle	115	19	96	37
Dead tissues and corpuscles	135	None	135	0
Living blood corpuscles	30	None	30	3
Starch grains	20	None	20	0
Blank clots	50	None	50	0
Total	996	150	846	181

of the various stages can be made while the cultures are alive. The reactions that occur in the clot appear to be the same with any of the tissues tried, and the description given will suffice for any of the preparations. The plasma in these tissue cultures, as is well

known, clots within a few minutes after the piece of living tissue has been added, and the imbedded tissue is firmly held by the fibrin net which forms in it. This fibrin net is of the same character as would be formed whenever plasma clots, and when observed under the microscope shows a typical fibrin net structure. This condition of the fibrin net continues in a high percentage of the preparations during the entire history of the culture (table I). Growth may occur in the imbedded tissue and the cells move freely out into the clot and yet no transformation of the fibrin net take place. The tissues of the mature frog are very inert as compared with the embryonic tissues of various animals, and in many cultures very little or no growth or movement of tissue cells occur. The heart muscle tissue of the frog forms, as a rule, quite a definite exception to the above statement, and in most cultures of this tissue a considerable cellular growth and movement will occur. However, whether there has been any growth of the imbedded tissue or not, some of the cultures in the course of two or three days will show that a change is taking place in the plasma clot surrounding the tissue. In the region lying nearest to the imbedded tissue, small fiber-like structures appear. At first these are quite small and fairly indistinct. They are of the same color as the fibrin net but have a different index of refraction, and they are large enough to be easily seen with the low power of the microscope. These fiber-like structures increase in size and number and ramify in all directions through the clot. They generally reach a high state of development by about the fifth to seventh day after the making of the preparation. Their development, however, will continue after this until in many cases at the end of two weeks almost the entire plasma clot has been changed into a new tissue which has a very different appearance and structure from that of the typical fibrin net. So far as has been observed, and a number of the cultures have been kept alive for periods exceeding three weeks before they were preserved, these fibers which form in the plasma clot are permanent. The fibers are consolidated to the greatest extent near the imbedded piece of tissue and radiate from that region in all directions to the periphery of the clot. To one studying the fiber formation in the living cultures it appears that they are direct outgrowths from the imbedded tissue. Experiments

which are given later in this paper show that such is not the case, however, and that the fibers are formed from the elements of the fibrin net. The clots in which the fibers have formed become very firm and solid and very unlike in this respect an unchanged plasma clot in which the typical fibrin net is present. This fact is particularly evident when one tries to remove a clot from the cover-glass for permanent preservation. The clots in which the fibers have formed can be handled roughly without injuring them at all. The regular plasma clots are very tender and have to be handled with the greatest care when they are removed from the cover-glasses in order to prevent their serious injury.

Microphotographs have been made of a number of the living cultures to show different stages of the fiber formation. Figure 1 is a microphotograph of a culture of liver tissue at a magnification of 33 diameters taken when the culture was three days old. A few of the small, newly formed fibers are to be noted in the plasma immediately surrounding the upper edge of the tissue. All the fibers present in this culture are not seen, inasmuch as it was impossible to bring them all into focus. This preparation, in the size and number of the fibers present, is typical of the average condition found in a two to three day culture in which the reaction has taken place. Numerous examples, however, of still greater development have been observed. It has been found that the development of the fibers is more rapid and prolific with spleen tissue than it is with any other of the tissues that have so far been used. Figures 2 and 3 are both microphotographs of spleen cultures taken when they were four days old. The magnification is 27 diameters in both instances and the photographs show clearly how striking and prominent the fibers are in the living preparations. Numerous blood corpuscles can be seen scattered through the plasma. Figure 4 is a microphotograph of a nine day culture which has been magnified 40 diameters and gives one a clearer view of the network of fibers which has formed in the plasma clot. It can also be noted that around the edge of the imbedded tissue a specialized part of the plasma has developed and the fibers appear to arise from this region. Such a region is not present in the clot at first and the early fibers appear to radiate from the edge of the imbedded tissue as shown in

figure 1. This region, in the older cultures, in which there is a transformation of the plasma, always occurs first in that region of the clot which is in direct contact with the imbedded tissue, and the changes which take place would therefore appear to be due to some action of the living tissue. The cultures from which figures 2 and 4 were taken were preserved, and a description of them is given later in this paper. In the cultures which are shown in figures 1 to 4 very little or no growth of the tissue cells took place, and, as has been noted, such is generally the case with most of the adult frog tissues. There are exceptions, however, and cultures have been under observation in which cell growth and movement as well as fiber formation could be noted. Such a condition is shown in figure 5, which is a photograph of a ten day liver culture. In this preparation a considerable number of fibers are to be noted in the plasma and others were present which are out of focus. To the left and below, it can be seen that a considerable outgrowth of cells from the tissue has occurred, among which are numerous large spindle cells. No relation is to be observed between these cells and the fibers. In cultures of heart muscle tissue a different condition is sometimes to be observed. An example of this is shown in figure 6, which is a photograph taken from a ten day culture of heart muscle tissue. In this culture quite a heavy fiber formation took place in the plasma surrounding the imbedded tissue during the first three or four days. Later spindle cells moved out from the tissue and followed the course of these fibers. This is the condition shown in the figure and several instances can be noted of the stereotropism of these cells. A similar condition has been observed in other cultures of this same tissue.[4]

STUDY OF THE PRESERVED CULTURES.

In order to make a histological examination of the fibers and their relations both to the imbedded tissue and the plasma clot, it was necessary to make and study mounted sections of the preserved cultures. A description of the methods used in preparing this material for microscopic study has already been given in a previous section of this paper.

In figure 7, which is a microphotograph of a spleen culture pre-

[4] Harrison, *Jour. Exper. Zoöl.*, 1914, *loc. cit.*

served at the end of the second day, an early stage in the fiber formation is to be seen. The fibrin net is for the most part unchanged, but a number of distinct fibers have been formed. These have arisen from the region of the clot lying nearest the tissue. Here the typical fibrin net structure has been partly lost, and it appears that there has been a consolidation or fusion of the fibrin elements to form a fibrous tissue. The evidence from the histological study of all the cultures apparently shows that the fibers have arisen through a transformation of the fibrin elements. However, the connection between the imbedded tissue and the fibers is so close in a great many instances that it is impossible to say from these observations alone that the fibers are not outgrowths of the imbedded tissue. Experiments, which are given in the following section of this paper, confirm the histological study of the cultures and make it certain that the new fibrous tissue arises from a transformation of the fibrin net. Figure 8 shows a microphotograph of a six day culture of spleen tissue at a magnification of 67 diameters. Figure 2, above, shows this same culture as it appeared while living at the time when it was four days old. In the two days intervening before it was preserved a considerable development of new fibers occurred, so that in figure 8 many more fibers are to be seen. This preparation is considered typical of a six day culture, and is worthy of careful consideration. The fiber formation shows a great advance over that pictured in figure 7. It will be noted that the fibrin net in the immediate vicinity of the imbedded tissue has been changed from the typical fibrin net structure into a dense, heavily staining tissue composed of wavy fibrous bundles in which, with sufficient magnification, the individual fibrils can be seen. From this dense fibrous area of the transformed fibrin net, bundles of fibers extend in all directions through the plasma clot. These bundles are heavier nearer the tissue and gradually grow smaller as they proceed out to the periphery and are finally lost in the undifferentiated fibrin net. They unite with other similar bundles of fibers at various places throughout their length. This condition can be seen in figure 9, which is a photograph of a portion of this culture magnified 667 diameters. In the upper part of this figure can be seen the dense fibrous tissue which lies next to the imbedded tissue, and proceeding from this area the wavy fibrous

bundles can be seen running to the less differentiated areas of the clot. These bundles can be seen to anastomose and interweave with each other to a considerable degree. In figure 10, which is also a photograph of a portion of this same culture magnified 1,000 diameters, the individual wavy fibrils which form the bundles can be seen. It is quite a difficult matter to show clearly the detailed structure of the fiber formation in the clots by means of microphotographs, especially with the higher magnifications. Accordingly in figure 11 a drawing of the same portion of this culture as is depicted in figure 10 is shown, which makes the structure of the transformed fibrin net still clearer. In the lower part of the figure a part of the imbedded tissue can be seen. In close contact with it is shown the heavy fibrous tissue with the bundles of fibers. These run to the periphery of the clot, gradually decreasing in size as they leave the region of the imbedded tissue, and are finally lost in the untransformed fibrin net. At the ending of the bundles, as well as in various other places shown in the drawing, an atypical arrangement of the fibrin elements can be noted. Apparently there is a fusion of these elements to form the fibrils and a grouping together of the fibrils to form the fibrous bundles. With the magnification used in this drawing ($\times 667$) it is possible in many cases to trace the fibrils from their point of origin in the fibrin net into the fibrous bundles. The fibrin net in the region of the imbedded tissue has become almost completely transformed from its typical structure, and only small areas of the clot remain which are unchanged. In the outlying regions of the clot large areas of the untransformed fibrin net can be seen. The complete absence of all isolated tissue cells is to be noted in this region of the culture.

Another preparation of interest is shown in figure 12. This microphotograph is from a nine day culture of spleen tissue. In figure 4 is shown a photograph of this culture made while living and just before its preservation. The fiber formation in this culture did not begin as early as it usually does and the development of the fibers is not as far advanced as found in some of the younger cultures as, for example, in figure 8, which is a six day culture. In figure 12 attention should be called to the part of the plasma clot which is lying in contact with the imbedded tissue. In this area the

fibrin net has become so changed from its typical structure and takes a stain so much like the tissue, that in the photograph it is difficult to make out the exact boundary line between the clot and the imbedded tissue. This transformed area is similar to that shown in figure 8, but the transformation is more complete. From the central part of this area great bundles of fibers are given off which run to the periphery of the clot. The greater portion of the clot in this culture still retains its typical net structure.

If conditions remain favorable the fiber formation continues in the plasma clot until practically the whole area has become transformed into a new fibrous tissue. The fibrin net disappears and the clot in some of the older cultures appears as a reticular tissue. The preparation shown in figure 13, which is a photograph at 100 diameters, presents an example of such a case. This culture has two pieces of liver tissue imbedded in the clot and was fourteen days old when preserved. The appearance shown in this figure is not exceptional, and a number of other preparations have been made which show as advanced a condition of fiber formation. It can be noted that practically the entire clot has become changed into what appears to be a reticular tissue. Figure 14, which is a microphotograph of a portion of this culture at 667 diameters, pictures one of the least differentiated areas, and from this it can be seen that the clot has been almost completely changed in its structure. In figure 17 a drawing of a portion of this preparation may be seen which shows the structure of the transformed fibrin net more clearly than is possible with photographs. At both the upper and lower edges of the drawing are shown a few of the cells of the two pieces of imbedded tissue. The clot lying between these two pieces of tissue has almost completely lost its characteristic fibrin net structure and instead we find a new fibrous tissue containing a great many bundles of wavy fibers. To all appearances this new fibrous tissue is identical with true reticular tissue as pictured and described by Mall.[5] It is held by many authorities that the fibrils of reticular tissue are identical with the fibrils of areolar tissue.[6] In some cases it can even be demonstrated

[5] Mall, F. P., Reticulated Tissue, and Its Relation to the Connective Tissue Fibrils, *Johns Hopkins Hosp. Rep.*, 1893, i, 171, plates 17 and 18.

[6] Schäfer, E. A., Text Book of Microscopic Anatomy, in Quain, J., Anatomy, 11th edition, London and New York, 1912, ii, pt. 1, 123, 124, figure 203.

that the fibrils of reticular tissue are in direct continuity with the white fibrils of the adjacent areolar tissue. In other words, the fibrils composing a reticulum may join together to form the bundles of fibers in the nearby areolar tissue. Such a relation between the fibrils and the fibrous bundles is also found in the tissue cultures where, as shown for example in figures 11 and 17, the fibrous bundles are composed of a great many fibrils, and in many instances these fibrils can be traced to where they leave the bundle and join the fibrin net. The bundles of fibers are typical of those found in areolar tissue. They grow smaller as they get away from the imbedded tissue, and in the outlying regions we find them breaking up and the fibrils of which they are composed continuing to form a typical reticular tissue.

THE ORIGIN OF THE FIBERS.

Although the study of the prepared cultures gives evidence that the new fibrous tissue arises through a transformation of the fibrin elements of the clot, nevertheless the close connection existing in the cultures between the imbedded tissue and the bundles of fibers makes it impossible to say that they have not arisen as direct outgrowths of the imbedded tissue. In order to settle the matter definitely it was found necessary to make several series of experiments with special types of cultures.

In the first series a large number of cultures were made in which the living tissues were imbedded in defibrinated serum instead of plasma. The method used in this series was to take the plasma from the frog by the usual technique and to divide it into two parts. One part was used in making the regular type of plasma cultures with living tissues imbedded. The other part of the plasma was defibrinated and used in making other similar cultures. The two sets of cultures were identical in every way except for the fact that some had the plasma while the others had the defibrinated serum, and these latter owing to the absence of the fibrin could not form any clot around the imbedded tissue. The results obtained from these experiments have always been the same, namely, the serum preparations have always failed to show any fiber formation, while in many of the parallel cultures in which the plasma was used the transformation of the

fibrin net occurred. Such a result indicates that the fibers are formed by a transformation of the fibrin net and not as outgrowths of the imbedded tissue.

In another series of experiments preparations were made as usual with plasma, but no tissue was imbedded. The plasma will clot of itself shortly after having been exposed to the air, and it was thought worth while to determine if fibers would form in these blank clots in which no tissue was present. Blank cultures were also made in which the plasma was coagulated by the addition of an extract obtained from fresh muscle tissue. This extract was obtained by mashing muscle tissue of the frog in a small amount of Ringer's solution and then centrifuging it to get rid of the blood corpuscles and small pieces of the tissue. By adding a drop of this extract to the plasma a very firm clot can be had in a few minutes. All the cultures of this series gave negative results and no transformation of the fibrin net occurred.

In a third series of experiments plasma cultures were made as usual, except that either living blood corpuscles, blood corpuscles which had been killed by heating, or starch grains were imbedded in place of living tissues. In the cultures in which the dead corpuscles and the starch grains were imbedded negative results were obtained and no change of the fibrin net occurred. On the other hand, in three of the thirty cultures in which the living blood corpuscles were imbedded a transformation of the fibrin net occurred. In figure 15 is shown, at a magnification of 100 diameters, a microphotograph of a culture in which living blood corpuscles were imbedded without any tissue. It can be noted that in the region in which the corpuscles are present quite a complete transformation of the fibrin net has occurred and a new fibrous tissue with bundles of long wavy fibers has been formed. A study of the fibers reveals no apparent difference in structure from those formed when living tissue is imbedded in the plasma. The outlying regions of the clot still maintain the typical fibrin net structure. The fact then that the transformation of the fibrin net can occur and the fibers arise in cultures in which only living blood corpuscles have been imbedded shows definitely that the fibers are not formed as outgrowths of the imbedded tissue, but they have been formed through a transformation of the fibrin net.

This result confirms that obtained from the histological study of the prepared cultures, which is given above.

FACTORS INVOLVED IN FIBER FORMATION.

The experiments just mentioned in the previous section showed that the new fibrous tissue arose by a transformation of the fibrin net. They also indicated that this transformation was caused by the presence of living tissues or cells in the plasma, inasmuch as cultures in which starch grains or dead blood corpuscles were imbedded, as well as the blank clots, all failed entirely to show any transformation of the clot. It was deemed wise to experiment further in an endeavor to find out if the transformation of the clot was dependent upon the presence of living tissues. To do this several series of parallel cultures were made use of. In order to make the method of experimentation clear it may be well to give the complete data of one such series of cultures. On February 14, 1914, the following cultures were made in the same manner from the same plasma and under identical conditions:

10 cultures of living muscle tissue.
10 cultures of living liver tissue.
13 cultures of living spleen tissue.
10 cultures of dead spleen tissue (the tissue had been heated to 60° C. in order to kill it).

Two days later fibers began to appear in the plasma clots of a number of the muscle and liver cultures, and fibers were also present in eleven out of the thirteen living spleen cultures. In the preparations in which the spleen tissue had been killed by heating no fibers ever appeared, and the plasma clots of these cultures never showed any change whatever from the fibrin net structure. Six parallel series of cultures, which were essentially the same as the series just described, have been made from time to time during the course of the work and the various dead tissues which were imbedded have been killed both by heating and poisoning. The results have always been the same, namely, no transformation of the fibrin net has ever occurred in any of the cultures in which the dead tissues were imbedded, while the reaction has always been present in some of the parallel living tissue cultures. In some of the preparations in which

the dead tissues were imbedded the extract from fresh muscle tissue was used to coagulate the plasma. The addition of the tissue extract did not in any apparent manner change the result previously obtained and no fiber formation has occurred in the clot of any culture except those in which either living tissue or blood corpuscles were imbedded. Attention should be called to another line of evidence which is in harmony with the above, and that is that in the living cultures in which the fiber formation takes place, the fibers appear first in the part of the clot which is in contact with the living imbedded tissue, and this part of the clot always shows the most complete transformation. In some cases the outlying regions of the clot, which are considerably removed from the influence of the tissue, retain the fibrin net structure while a complete transformation of the clot may take place near the imbedded tissue (figure 12). The results from all the experiments strongly suggest that the reaction which causes the fiber formation is due to some property of the living tissues which have been imbedded. The author, however, does not regard the tests so far made as exhaustive enough to say with absolute certainty that the transformation of the clot can only take place under the influence of living tissues or cells. There is the possibility that the reaction is one which will occur under the influence of other conditions which as yet are not known. Whether or not such is found to be the case the essential thing to be noted is that the imbedded living tissues are by themselves able to bring about a radical transformation of the fibrin elements of the clot and the consequent formation of a new fibrous tissue which, in appearance at least, is a typical connective tissue.

It has been found possible by mechanical means, such as pulling and twisting the clots with dissecting needles, to cause the fibers to form very rapidly in the clots in which the living tissues have been imbedded.[7] In such cases the piece of living tissue is placed in the

[7] An account of some early work by Leo Loeb along this line that is suggestive is given by Adami, J. G., The Principles of Pathology, 2d edition, Philadelphia and New York, 1910, i, 40. To quote: "When a drop of uncoagulated lymph is placed between two glass slides, the mere act of pulling one slide over the other leads to the appearance of fibrils, which grow in length and bulk; which, like those of connective tissue, are not only intracellular, but actually traverse cell bodies situated in their path; which show themselves first in imme-

drop of plasma as usual. After the clot has formed around the imbedded tissue the formation of the fibers can be aided by manipulating the clot with needles and thereby exerting tension in various directions. Such a preparation is shown in figure 16 in which a piece of spleen tissue was imbedded and the clot manipulated with needles. The culture was then sealed as usual, left over night, and preserved the next morning. It will be noted that the fibers are very irregular and formed in the various regions following the path of the needles through the clot. Such a condition is different from that shown in the previous figures in which the fibers were allowed to develop slowly in an undisturbed clot. In either case the structure of the fibers appears to be the same and to have resulted from a consolidation or fusion of the elements of the fibrin net. In the disturbed clot these processes are evidently hastened by the introduction of the mechanical factor. Many cultures have been made during the course of the work in which the clots were disturbed. Also the effect has been noted of imbedding killed tissues and then disturbing the clots. So far such cultures have always failed to show the fiber formation except that, if the plasma is caused to clot by the addition of a little of the fresh tissue extract and then the clot is disturbed, there occasionally appears to be an attempt at fiber formation. Possibly it might be expected that such a result would be obtained, since it is probable that the fresh tissue extract contains the constituents of the living tissue which are responsible for the transformation of the clot.

Two points may be noted as a result of these experiments: first, the evidence at hand strongly indicates that the presence of living tissue or of living cells is necessary to cause a transformation of the plasma clot; and, second, that when either of these is present the introduction of a mechanical factor may aid in a more rapid formation of the fibers.

The fact that mechanical means could be used to assist in the formation of the fibers suggested to the author the possibility that

diate connection with these cells, the cells, as we now hold, liberating an enzyme that determines the modification of the more soluble protein into a precipitated or coagulated modification. But the lines of the precipitation are evidently along the lines of strain."

the fibers in the regular cultures were due to the action of living cells which had moved out from the imbedded tissue or that were present in the plasma; in other words, that perhaps the fibers marked the paths of the various isolated cells which had become separated from the tissue or of cells which were present in the plasma, such as blood corpuscles, lymphocytes, etc. In most of the cultures it can readily be observed that numerous blood cells are present in the plasma clots. In actively growing cultures also a great many cells move from the imbedded tissue to various regions of the clot. The cultures of heart muscle tissue of the adult frog show this to the greatest advantage, and in such cultures many instances can be noted in which the fibers are in close relation to the isolated spindle cells. Such a culture is shown in figure 18, which is a microphotograph at 100 diameters of a four day culture. Numerous spindle cells can be seen in the fibrin net to the right of the imbedded tissue. The path of these cells in their movement from the tissue to their present position can be traced by the appearance of the fibrin net. In such a culture it appears probable that the fibers which have been formed have arisen partly at least as a result of the action of the cells in forcing their way through the fibrin net. In the cultures of heart muscle tissue which have been under observation the formation of fibers is generally associated with the spindle cells which have moved through the clot from the imbedded tissue, whereas in cultures of spleen and liver tissue in which no growth or movement of tissue cells has taken place the fibers arise directly from that region of the plasma clot which is in contact with the imbedded tissue. A comparison of figure 8 with figure 18 will make this statement clear. In the first figure with spleen tissue, the fibers have arisen in close connection with the imbedded tissue, while in figure 18, with the heart muscle tissue, the fibers have, in general, arisen in close contact with the individual cells that have wandered out from the tissue, and there are very few fibers which are in contact with the imbedded tissue. In figure 19, which is a microphotograph at 667 diameters of a portion of figure 18, a better idea can be obtained of the relations existing between the spindle cells and the fibrous bundles in these cultures of heart muscle. The figure shows one of the spindle cells moving through the plasma clot. Below its path can be traced by

the appearance of the fibrin net which has been transformed into a fibrous tissue. Long fibrous bundles also radiate out laterally from the region of the cell. Above the fibrin net still retains its typical structure. In figure 20, which is a microphotograph of an eight day heart muscle culture at a magnification of 667 diameters, a condition is shown which is typical of that found in the older cultures of this tissue. The part of the clot lying next to the imbedded tissue and through which the cells have wandered has become almost entirely transformed into the bundles of fibers and with the cells lying between these a tissue is formed which is entirely similar, in its appearance at least, to the regular connective tissue of the frog. It is generally supposed that the fibroblasts which wander into a fibrin clot from the injured tissues during wound healing digest the fibrin and then form the new connective tissue fibers intracellularly. Such, however, is not the case with these fibers which have formed from the elements of the fibrin net. They are not digested by the tissue cells which have wandered into the clot, but, on the other hand, they are acting as permanent fibers and have combined with the tissue cells to form a connective tissue.

In the cultures of heart muscle tissue, then, the results indicate that the tension exerted by the isolated spindle cells in their movements through the plasma clot plays a part in the fiber formation. However, it can be shown that the movements of isolated tissue cells are not responsible for the formation of fibers in all the cultures, for the same reaction will take place in cultures in which there are no isolated cells present in the clot, and, therefore, the transformation which occurs must be due to the action of the living tissue as a whole without the aid of any apparent mechanical factor. In cultures of adult frog spleen and liver there is, in general, very little, and in some cases no growth or movement of tissue cells, and yet it is in cultures of these tissues that the most prolific fiber formation takes place. With care it has been found possible to make a culture in which the plasma is also free from blood corpuscles. Such a culture, providing there is no outgrowth of cells from the imbedded tissue and that a transformation of the fibrin clot has occurred by which the new fibrous tissue has been formed, demonstrates that the imbedded tissue can bring about the reaction without the aid of iso-

lated cells. A number of such instances have been noted during the course of the work and a microphotograph of such a culture is shown in figure 13, in which preparation the clot showed an almost complete absence of isolated cells and no movement of tissue cells occurred. Notwithstanding the absence of isolated cells in the clot an almost complete transformation of the fibrin net took place. Such a result makes it clear that the imbedded living tissue is, without the aid of isolated cells or of any other apparent mechanical factor, able to bring about the formation of the fibrous tissue from the fibrin elements of the clot.

THE NATURE OF THE NEW FIBROUS TISSUE.

In an endeavor to determine the nature of the new fibrous tissue, which arises in the cultures through a transformation of the fibrin elements of the clot, experiments with various specific stains have been made. Also the new tissue has been subjected to digestion and acid tests. It was hoped that these experiments would show definitely whether or not the fibers composing the new tissue were fibrin in character, although apparently identical in their appearance, structure, and function with the regular collagenous fibers of connective tissue.

Staining Reactions of the New Fibrous Tissue.—Two stains have been made use of, both of which are supposed to be specific to differentiate between fibrin and connective tissue; namely, Mallory's connective tissue stain modified according to Mall,[8] and Van Gieson's picrofuchsin stain. Early in the work it was found that with the Mallory stain the fibers formed in the fibrin net were stained a blue color which very closely resembled the color of the regular connective tissue fibrils when stained in the same manner. With this stain fibrin is supposed to stain red in contrast to the blue of the connective tissue. However, the fibrin of frog plasma stains a purplish blue instead of a red with this stain. The differentiation, though between the purplish blue of the undifferentiated fibrin net and the almost ultramarine blue of the new fibers, is very clear. All the microphotographs that are shown in this paper were made from

[8] Mall, F. P., On the Development of the Connective Tissues from the Connective-Tissue Syncytium, *Am. Jour. Anat.*, 1901-02, i, 338.

preparations which were stained with the Mallory stain, and from these it can be seen that the differentiation is very sharp between the fibrin net and the fibers which form in it. In comparison with the regular connective tissue fibers which can be studied in the imbedded tissues of the cultures it can be said that with the Mallory stain there is in most instances very little difference in color between them and the fibers which have been formed by the transformation of the plasma clot. The regular connective tissue fibers stain a perfect ultramarine blue, while the fibers which form in the clot in the same culture are generally a little darker in color and a little less clear. In short it may be said that when this stain is used the regular connective tissue fibers and the fibers formed in the clot stain so nearly the same that there is no real difference in their appearance. This result, however, is not obtained when the cultures are stained with Van Gieson's stain. This stain colors connective tissue fibers red and other tissues yellow. Using this stain on the preparations it has, thus far, been found impossible to obtain any differentiation between the undifferentiated plasma clot and the fibers formed in it, the entire clot with the fibers taking a yellow stain. The fibers can be clearly seen in the clot of a culture thus stained, but they have the same yellow color as the parts of the clot which have not been transformed, in distinction to the red color of the regular connective tissue fibers. With this stain, therefore, the evidence is that the fibers formed in the clot retain their fibrin character. A combination of the two stains has been used in a number of instances. In this case the culture is stained first with the Mallory stain, then partially decolorized with acid alcohol, and restained with Van Gieson. Preparations treated in this manner show a clear differentiation between the fibers in the clot which stain a light green, as do also the regular connective tissue fibers in the imbedded tissue, and the remainder of the clot which takes a pinkish stain.

Digestion and Acid Tests with the New Fibrous Tissue.—The fact that fresh fibrin is easily dissolved by digestion with pancreatin or by the use of a weak acid, whereas connective tissue fibers are not so affected, was used as a basis for determining the nature of the fibers formed in the clot. The method of experimentation was as follows: In a culture in which the fibers were present in the clot, the cover-

glass, to which were attached the clot and the imbedded tissue, was removed and immersed in the pancreatin solution at 37° C. or in a dilute solution of acetic acid. Cultures ranging in age from five to fourteen days have been experimented with in this manner. The result, with either of the tests, has always been that within a few hours the entire clot containing the fibers completely disappears leaving the imbedded tissue free. Such a result apparently indicates that the fibers formed in the clot remain fibrin in character. The tests, however, are not conclusive, because of the intimate relations existing between the fibrin net and the fibers which form in it. The destruction of the fibrin net either by digestion or with a weak acid naturally causes a breaking down of the fibrous bundles which are formed in it and a scattering of the individual fibrils, inasmuch as they are not attached to the imbedded tissue and therefore their only support is the fibrin clot in which they are formed. Thus far it has proved to be a difficult matter to get a test that can be used in these tissue culture preparations that will definitely distinguish between fibrin and connective tissue fibers. Mall[9] mentions the great difficulty he found in using the digestion and acid tests in the study of the early differentiation of the connective tissue fibers. He states that the results from various tests contradicted each other, so that he hesitated to draw any conclusions from them. Difficulties of the same kind have been encountered in this work, and the author does not lay great weight upon the results of the tests so far made as showing definitely whether or not the fibers formed are really connective tissue fibers.

In brief, the structure and function of the new fibrous tissue and the appearance with Mallory's stain all indicate that it is a true connective tissue. On the other hand, the Van Gieson stain and the acid and digestion tests give evidence that the new fibrous tissue retains the fibrin character of the fibrin net from which it formed. Work along various lines is in progress and it is hoped that as a result the true character of the new fibrous tissue can be definitely settled.

[9] Mall, *Am. Jour. Anat., loc. cit.*, p. 339.

DISCUSSION.

With the results of these experiments at hand it may be well to discuss briefly the significance of the changes which take place in the cultures of adult frog tissues. It is evident that in the reaction which occurs in the plasma clot, involving as it does a transformation of the fibrin net into a new fibrous tissue, we have a process which must be fundamental in wound healing. A wound under the most favorable conditions for healing becomes quickly filled with the fluid plasma and lymph which pour into it from various sources. This quickly coagulates and a fibrin net is formed.[10] Under the influence of the living injured tissues, and aided to some extent by the tension exerted on it through the movements of the tissues, this mass of fibrin soon loses its typical structure. As shown in this paper, the fibrin elements fuse or consolidate to form fibrils, and these unite to form long wavy fibrous bundles which freely intertwine and anastomose and ramify through the entire clot. In a short time there has arisen, in place of the fragile and easily destroyed plasma clot, a new fibrous tissue which is tough and resistant and which functions for the time, at least, as a regular connective tissue. The transformation of the clot also causes a shrinkage, and the wound space is thereby lessened, the edges of the wound are held firmly in place, and a support is formed for the outgrowth of the tissue cells.

From the work presented in this paper it also appears that in wound healing, at least, there is good reason for believing that this new fibrous tissue which arises by a direct transformation of the fibrin clot, without intracellular action, remains as a permanent connective tissue. Hertzler[11] has already put forth such a view which he bases on results obtained from wound healing. He shows

[10] For a summary of the part played by fibrin in wound healing, see Marchand, F., Der Process der Wundheilung, Stuttgart, 1901, 52–55. The fact that the fibrin elements in wound healing consolidate to form fibers is also shown; see Marchand, *loc. cit.*, pp. 245–255, figures 64–67.

[11] Hertzler, A. E., Pseudoperitoneum, Varicosity of the Peritoneum and Sclerosis of the Mesentery. With a Preliminary Note on the Development of Fibrous Tissue, *Jour. Am. Med. Assn.*, 1910, liv, 351; The Development of Fibrous Tissues in Peritoneal Adhesions, *Anat. Rec.*, 1915, ix, 83.

that the fibrous bundles are present in the clots in wound healing and also that they will take a specific connective tissue stain a short time after they are formed. Hertzler believes that the fibers arise through some chemical action. Although there is considerable support for the view that the connective tissue fibers, in general, arise through intercellular action,[12] the idea put forth by Hertzler that they arise by a transformation of the fibrin elements of the clot does not appear to have received any support. In the present paper it has been possible to show by the use of tissue culture methods that under the influence of living adult frog tissue a transformation of the fibrin elements of the clot may give rise to a new fibrous tissue which in appearance, when stained with Mallory's stain, structure, and function is identical with regular connective tissue. The experiments which have so far been made use of in an endeavor to determine the nature of the new fibrous tissue have failed to settle the question definitely, and until it is possible to get a test that will determine the question no definite statement regarding the nature of the tissue should be made. The work with the older cultures shows that the new fibrous tissue remains permanently (figure 17), and also that it is not digested by the tissue cells which wander into it, but, on the other hand, the two combine to form a typical areolar tissue (figure 20). There appears to be no reason for believing that the fibers of the new tissue would ever be replaced by other fibers formed intracellularly. Personally, from the study of the living and preserved cultures that has been made, the author feels that there is good ground for the belief that the fibers which have formed in the clot through a transformation of the fibrin elements are identical with those found in areolar and reticular tissues. Whether or not the work which is now being carried on, which embraces a study of the origin of the connective tissue fibers in wound healing and also in embryonic development, bears out this belief, as suggested above, a direct transformation of the clot into the new fibrous tissue such as has here been definitely shown must be a process which is of the highest value in wound healing.

[12] For an account of the various views held regarding the origin of the connective tissues, see Schäfer, *loc. cit.*, pp. 116–122. Adami, *loc. cit.*, p. 429.

SUMMARY.

In living cultures of various kinds of adult frog tissues, which have been made according to the hanging drop method, there occurs, in many cases, a transformation of the plasma clot by which it becomes entirely changed from a typical fibrin net both in appearance and structure.

The changes in the fibrin net generally begin when the culture is from two to three days old. During these changes it appears that the elements of the fibrin net fuse or consolidate, and as a result a great number of fine wavy fibrils are formed which unite to form wavy bundles of fibers, and these freely intertwine and anastomose as they ramify through the area of the plasma clot. The transformation of the fibrin net occurs first in the region of the clot which lies next to the imbedded tissue, gradually extends to the distal regions of the clot, and in time—as a rule in about two weeks—the entire plasma clot becomes changed from the fibrin net into a structure which to all appearances is identical with regular connective tissue. Photographs of both living and preserved cultures have been made to show the course of the transformation of the plasma clot and the development of the fibers.

Experiments have been made which show that the fibers which are formed are not outgrowths of the imbedded tissue. Also they are not formed by an intracellular action, but arise directly by a transformation of the fibrin elements of the plasma clot.

Experiments have been made which indicate that the transformation of the fibrin net will not occur unless it has come under the influence of living tissues or of living isolated cells. However, mechanical means, such as exerting tension on the clot with needles, may hasten the formation of the fibers. Also, in some cultures, movements of living isolated cells appear to aid in the formation of the fibers. The living tissues alone, however, are able to cause the fibers to form without the aid of any apparent mechanical factor. This is shown by cultures of various tissues in which no cell movement occurs and in which the plasma clot is undisturbed and yet a prolific formation of fibers may take place.

Experiments have been made in order to determine the true nature

of the transformed plasma clot and to see if the new fibrous tissue were still fibrin in character. The results that have so far been obtained from these tests have not been entirely conclusive and leave the question unsettled.

The transformation of the fibrin net results in a shrinkage of the clot. It also becomes very tough and resistant to injury and, therefore, entirely different from the fragile and easily destroyed fibrin net when in its original condition. It is believed that such a reaction must play an important part in wound healing. A study of the relation between connective tissue fibers formed in wound healing and in embryonic development to the fibers formed in the plasma clot is being made, and the results will appear in a later paper.

EXPLANATION OF PLATES.

All of the figures shown are microphotographs[13] with the exception of Nos. 11 and 17 which are drawings. Figures 1 to 6 inclusive were taken from the living cultures. The remainder of the figures were taken from the preserved cultures and were made with a Bausch and Lomb microphotographic apparatus equipped with a Zeiss microscope and apochromatic lenses.

PLATE 31.

FIG. 1. Three day culture of liver tissue. × 33. From the upper edge of the imbedded tissue a number of small fibers can be seen projecting into the plasma clot.

FIG. 2. Four day culture of spleen tissue. × 27. A prolific growth of fibers is shown. This culture was preserved. See figures 8 to 11.

FIG. 3. Four day culture of spleen tissue. × 27. Two pieces of imbedded tissue are shown, from both of which a great many of the fibers are given off into the plasma. The pieces of tissue were not connected at first. Later they became connected by the fibrous formation in the clot and then cells wandered out on these fibers as shown in the figure. Numerous blood corpuscles are present in the clot. Also many small light areas are noticeable in the clot. These are the result of condensation on the glass slide.

FIG. 4. Nine day culture of spleen tissue. × 40. Fibers are shown at a higher magnification. Note specialized area of the clot around the imbedded tissue from which the fibers arise. This culture was preserved. See figure 12.

FIG. 5. Ten day culture of liver tissue. × 33. A considerable cellular movement from the imbedded tissue has occurred, as well as a prolific fiber formation.

FIG. 6. Ten day culture of heart muscle tissue. × 33. A heavy cellular growth and fiber formation have occurred. At the lower edge of the tissue cells can be noted which are apparently travelling along the fibers.

[13] The author is glad of this opportunity to express his thanks to Professor Alexander Petrunkévitch for his advice and assistance in making some of the microphotographs.

PLATE 32.

FIG. 7. Two day culture of spleen tissue. × 100. An early stage in the fiber formation is shown in this figure. A small area of the clot which is in contact with the imbedded tissue is becoming transformed. The rest of the clot retains the typical fibrin net structure.

FIG. 8. Six day culture of spleen tissue. × 67. A heavy fiber formation is to be noted in the clot. A dense fibrous tissue has been formed in the clot almost entirely around the imbedded tissue, and from this area the fibers radiate to the outlying regions. See figure 2; also figure 11 which shows a drawing of a part of this region.

FIG. 9. A part of the culture shown in figure 8. × 667. A portion of some of the bundles of wavy fibers are shown and also some of the untransformed clot. Above can be seen a part of the dense fibrous tissue which surrounds the imbedded tissue.

FIG. 10. A part of the culture shown in figure 8. × 1,000. The fine fibrils which compose the bundles of fibers can be noted, as well as some of the untransformed fibrin elements of the clot.

PLATE 33.

FIG. 11. A drawing of a part of the culture shown in figure 8. × 667. Below a small portion of the imbedded tissue is shown. Lying in close contact with this is the dense fibrous tissue which has formed in the clot. Bundles of fibers, which compose this area, radiate to the outlying regions of the clot, gradually grow smaller, and finally end in the untransformed fibrin net. The elements of the net appear to fuse together to form the fibrils which in turn unite to form the fibrous bundles. No isolated cells are present in this region of the culture.

PLATE 34.

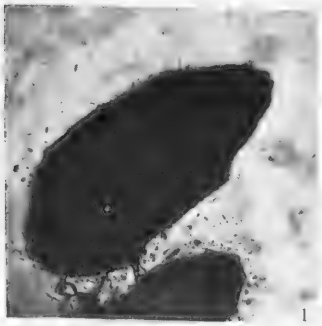
FIG. 12. Nine day culture of spleen tissue. × 100. The portion of the clot lying in contact with the imbedded tissue has become changed from the fibrin net to a dense fibrous tissue from which large bundles of fibers are given off. The transformation has been so complete in this region that it is difficult to make out the boundary between the clot and the imbedded tissue. See figure 4.

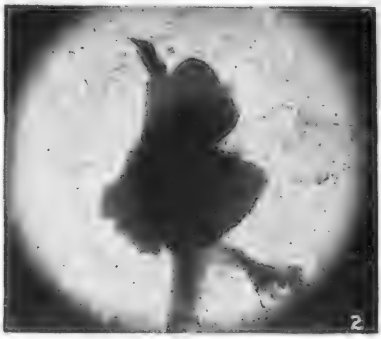
FIG. 13. Fourteen day culture of liver tissue. × 100. Two pieces of the imbedded tissue are shown. The portion of the clot lying between them has become almost completely transformed into what gives every appearance of being a reticular tissue. See figure 17, which shows a drawing of this same culture.

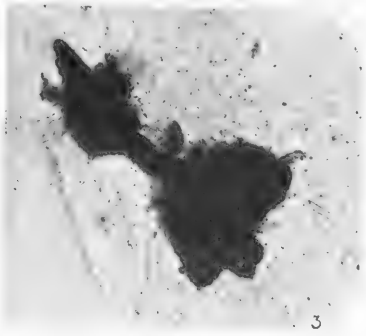
FIG. 14. A portion of the new fibrous tissue formed from the clot of the culture shown in figure 13. × 667.

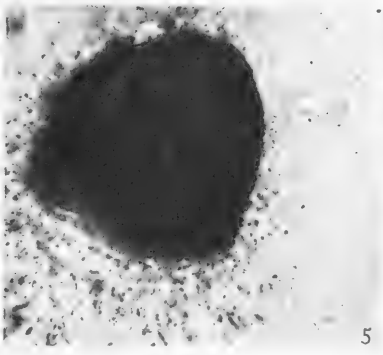
FIG. 15. A preparation in which living blood corpuscles were imbedded in place of a piece of tissue. × 100. The fibers have formed in the region in which the corpuscles were imbedded.

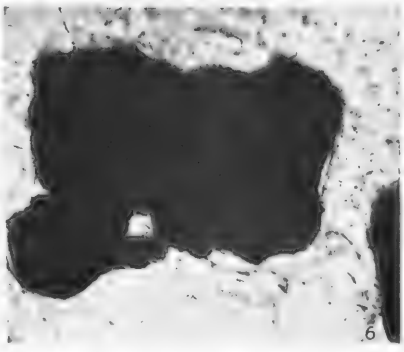
FIG. 16. A preparation in which the clot was disturbed by exerting tension with needles. × 100.



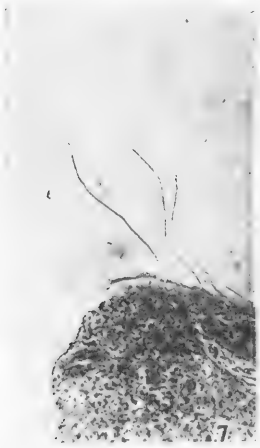


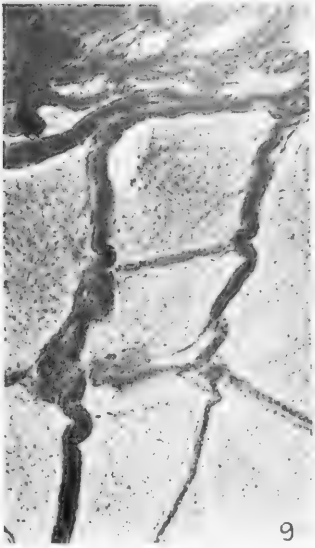






(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)

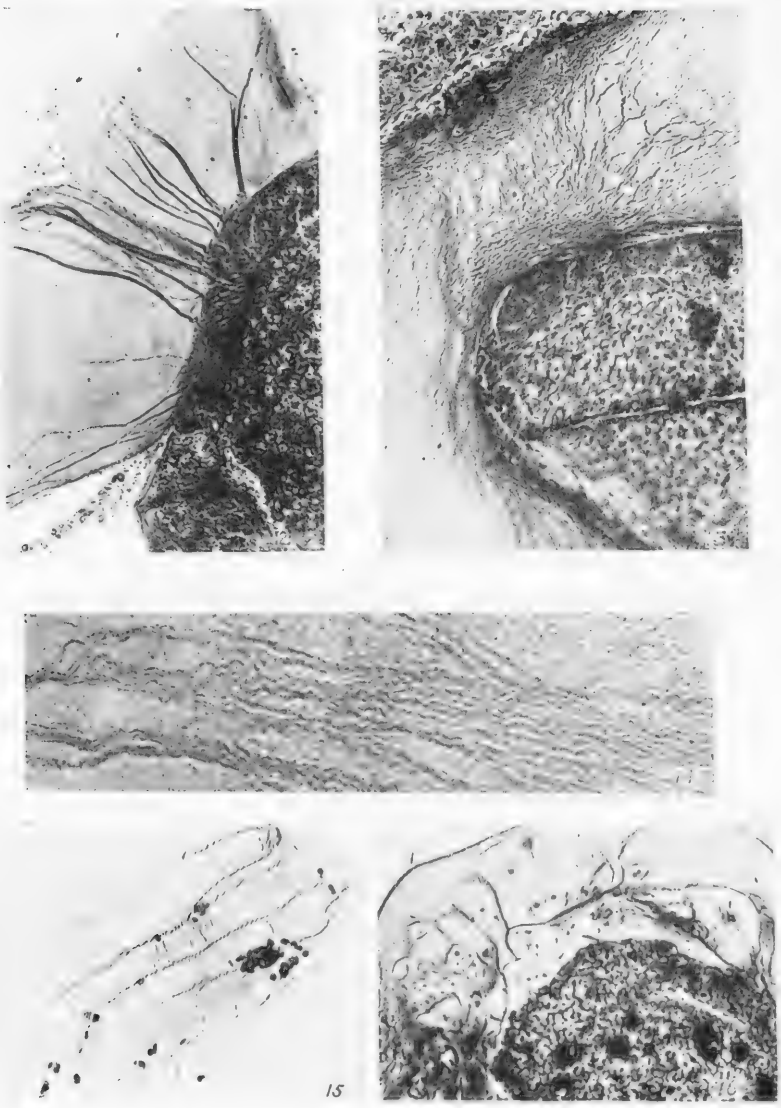




(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)

FIG. 11.

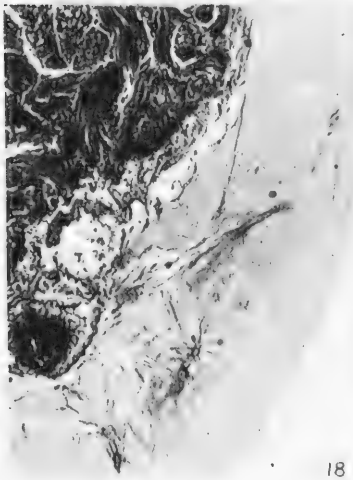
(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)



(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)

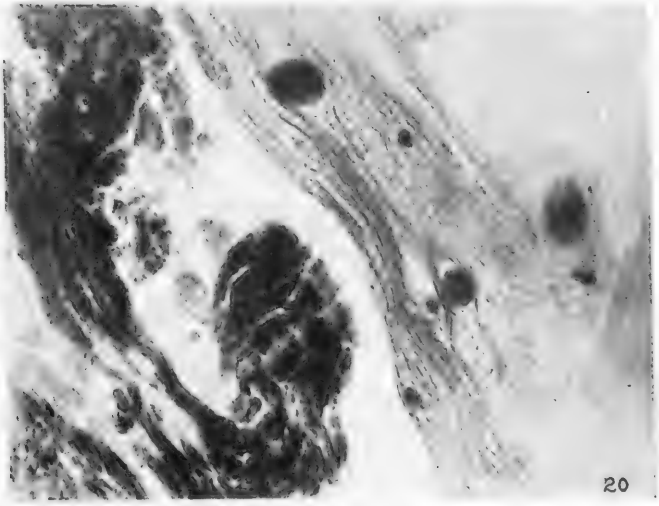
FIG. 17.

(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)



18

19



20

(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)

PLATE 35.

FIG. 17. A drawing of a part of the culture shown in figure 13. ×667. It can be seen that practically the entire clot has been transformed into what appears to be a reticular tissue. Only very small areas are present in which a fibrin net appears. The most complete transformation of the clot lies nearest to the imbedded tissue.

PLATE 36.

FIG. 18. Four day culture of heart muscle tissue. ×100. The fibers have formed in close connection with the cells which have wandered out from the tissue.

FIG. 19. A part of the culture shown in figure 18. ×667. One of the spindle cells is shown. It has moved out from the imbedded tissue and its path can be traced by the appearance of the fibrin net, which has, in the vicinity of the cell, been largely changed into a fibrous tissue.

FIG. 20. A portion of an eight day heart muscle tissue culture. ×667. The clot lying in contact with the imbedded tissue has become changed into the new fibrous tissue with its bundles of fibers, and these with the tissue cells which have wandered out from the imbedded tissue give a typical areolar tissue structure.

THE NATURE OF ANAPHYLATOXIN.

Studies on Immunity. II.*

By J. BRONFENBRENNER, Ph.D.

(From the Pathological and Research Laboratories of the Western Pennsylvania Hospital, Pittsburgh.)

It was shown by Friedberger (1) that the specific union between antigen, antibody, and complement is accompanied by the production of anaphylatoxin. If the mechanism of the Abderhalden reaction rests, as was suggested by my experiments (2), on the specific union of substratum and the specific fraction of immune serum, and if the presence of the complement is essential for the reaction to take place, there must appear during the reaction toxic products similar to those of Friedberger.

Experiments performed with this in view showed that the Abderhalden reaction is accompanied by the formation of highly active anaphylatoxin. These experiments were performed as follows.

The serum of three pregnant guinea pigs was mixed and placed in two centrifuge tubes, 6 c.c. in each, and about 3 gm. of boiled placenta tissue was added to

TABLE I.

Date.	Animal No.	Weight.	Dose.	Material injected.	Results.
Apr. 10...	160	250 gm.	3 c.c.	Supernatant fluid resulting from digestion of pregnant serum with placenta.	Death in 3 min.
Apr. 10...	162	255 gm.	2 c.c.		Death in 3 min.
Apr. 10...	163	255 gm.	1 c.c.		Death in 3 min.
Apr. 10...	165	260 gm.	0.5 c.c.		Grave symptoms. Recovery in 30 min.
Apr. 10...	167	250 gm.	2 c.c.	Supernatant fluid resulting from digestion of male serum with placenta (control).	No symptoms.
Apr. 10...	168	250 gm.	4 c.c.		No symptoms.
Apr. 10...	169	245 gm.	5 c.c.	Pregnant serum not having been digested with placenta (control).	Slight dyspnea.

* A preliminary communication was presented at the meeting of the Pennsylvania State Medical Society, September 22, 1914. Received for publication, December 29, 1914.

one of them. At the same time 6 c.c. of the serum of a male guinea pig was put in another centrifuge tube with 3 gm. of boiled human placenta tissue. All these tubes were placed in the incubator for sixteen hours, and at the end of this time the tubes were centrifuged, the serum was separated, and tested for toxicity by intravenous injection into normal male guinea pigs of about 250 gm. The results are shown in table I.

Parallel with this series, the same sera, both pregnant and male, were tested by the regular Abderhalden method with the result shown in table II.

TABLE II.

Pregnant guinea pig serum.		Male guinea pig serum.	
Serum 1.5 c.c. Placenta 0.5 gm.	Serum 1.5 c.c. No placenta	Serum 1.5 c.c. Placenta 0.5 gm.	Serum 1.5 c.c. No placenta
16 hrs. at 37° C. in dialyzing thimbles.			
Ninhydrin test +	Ninhydrin test −	Ninhydrin test −	Ninhydrin test −

As the above experiments suggest, the appearance of dialyzable substances on which the Abderhalden reaction depends, and the toxicity of the serum resulting from the interaction between the serum and the placenta tissue in the test-tube run parallel, and must, therefore, depend on some specific mechanism very similar or even identical in both cases. Since, however, it was suggested by the experiments reported previously (3) that the dialyzable split products appear in the Abderhalden test as a result of autodigestion of the serum, the results of the above experiment suggest that the appearance of anaphylatoxin in this experiment may be due to autodigestion of the serum. This conclusion would open the question of the mechanism of the formation of anaphylatoxin in general.

There exist at present two main theories explaining the origin of anaphylatoxin: one is a chemical theory developed by Friedberger (1910–1914); the other, first adopted by Doerr (4), explains the phenomenon on a physical basis. In his earliest experiments Friedberger (5) obtained from normal guinea pig serum which had been allowed to stand for some time with the washed specific precipitate (formed by rabbit serum immunized against sheep serum with the serum of the latter), very strong poisons which killed the guinea pigs instantly with symptoms of acute shock. He named the poisonous substance anaphylatoxin, and assumed that it arose from the digestion of the specific precipitate by the ferments of normal guinea pig serum. Later it was shown by Friedberger (6) and his pupils (7) that the anaphylatoxin may be derived also from different bacteria and other proteins by their incubation with normal sera without concurrence of specific antibody, and that the physiological action of the poisons thus obtained is similar

to that of the chemical poisons obtained previously by Vaughan and Wheeler (8). But Friedberger obtained them from various proteins by the action of proteolytic ferments of normal serum, whereas Vaughan and Wheeler obtained them from the same proteins by means of chemical agents.

According to the physical theory, the source of anaphylatoxin is not the protein of the antigen but the serum itself, which becomes toxic as a result of the removal of some substances from it, by means of physical adsorption. Ritz and Sachs (9), for instance, assume that the normal toxicity of the serum is masked by the presence of antagonistic substances which may be removed by adsorption. The recent experiments of Bordet (10) and those of Nathan (11) and Mutermilch (12) in which the substances causing the formation of anaphylatoxin by incubation with guinea pig serum, namely, agar, starch, and kaolin, are not of a protein nature, seem to give strong evidence in favor of the physical theory. The question, however, is not yet settled, as Friedberger (13) objected to the results obtained with agar and starch on the basis that these substances contain a small percentage of protein impurities and thus still may furnish the substratum for digestion.

If the interpretation given to the results obtained in the experiment above is correct, that is, if the toxicity of the end-product in the Abderhalden test is due to the autodigestion of the serum, the findings of Mutermilch can be easily explained, since it was shown in my previous publication (3) that the addition to the serum of kaolin causes the autodigestion of the serum.

However, since Friedberger failed to confirm the findings of Mutermilch, it was deemed necessary to establish first whether the digestion of the serum with kaolin is followed by the appearance of anaphylatoxin.

TABLE III.

Date.	Animal No.	Weight.	Dose.	Material injected.	Results.
Apr. 20...	311	255 gm.	1.5 c.c.	Serum treated with kaolin and incubated at 37° C.	Grave symptoms. Found dead next morning.
Apr. 20...	312	250 gm.	1 c.c.	Serum treated with kaolin and incubated at 37° C.	Grave symptoms. Recovery in 15 min.
Apr. 20...	313	255 gm.	4 c.c.	Untreated serum.	No symptoms.
Apr. 20...	314	250 gm.	3 c.c.	Serum treated with kaolin immediately after removal from ice.	No symptoms.

Freshly drawn guinea pig serum was placed in a centrifuge tube and immediately after the addition of an excess of sterile kaolin the tube was placed on ice, and, as soon as the kaolin settled out, a new portion of kaolin was added. Special care was taken that the kaolin should not be lumped, and that the particles of kaolin should be dispersed as uniformly as possible in the serum before they

were allowed to settle. After the serum had been treated in this manner three times, which took over nine hours, the contents of the tube were centrifuged, the serum was separated, and transferred to the incubator. After sixteen hours' incubation the serum was injected intravenously into normal guinea pigs weighing about 250 gm., and was found to be toxic, as shown in table III.

Since kaolin is not soluble, it is evident that the toxicity of the supernatant fluid is due to changes in the serum. The possible objection that particles of kaolin may be suspended in this serum, and thus cause harm mechanically or chemically, is obviated by the fact that, as the experiment shows, the same serum, although toxic after incubation in the thermostat, is not toxic immediately after removal from kaolin.

As to the nature of the changes which may have taken place in the serum, my experiments show that the contact with kaolin deprives the serum of its antitrypsin and that subsequent incubation at 37° C. is followed by the autodigestion of the serum.

DOES PLACENTA FURNISH TOXIC PRODUCTS IN THE ABDERHALDEN REACTION?

In view of the results discussed above, the suggestion that the anaphylatoxin formed during the Abderhalden test originated from the serum seems to be strengthened. However, since in one instance the serum is digested with kaolin, which by itself cannot be the source of digestible material, whereas in the other instance serum is combined with a placenta tissue, and especially since it is asserted by Abderhalden that in these conditions of the experiment placenta is digested, it was deemed necessary to arrange the experiment so as to avoid even the possibility of the digestion of placenta before it would be possible to determine the source of the toxic material in this case. This was attempted in the following experiment (table IV).

12 c.c. of pregnant guinea pig serum were placed on ice in a tube with about 5 gm. of boiled human placenta tissue. Sixteen hours later the contents of the tube were centrifuged and the supernatant fluid[1] was separated from the sediment. 8 c.c. of this fluid were transferred into another tube and placed in the incubator (37° C.) for sixteen hours. At the end of this time the contents of

[1] Serum treated in this way, as was shown before, is deprived of its specific constituents as well as of the natural antitryptic inhibition, and if placed at this stage in the incubator it undergoes autodigestion.

the tube were tested for toxicity by the intravenous inoculation in normal male guinea pigs. The remaining 3 c.c. of supernatant fluid collected after centrifugation were tested for toxicity immediately after centrifuging.

TABLE IV.

Date.	Animal No.	Weight.	Dose.	Material injected.	Results.
Apr. 13...	200	250 gm.	3 c.c.	Supernatant fluid before incubation.	No symptoms.
Apr. 14...	202	253 gm.	3 c.c.	Supernatant fluid after incubation at 37 °C. for 16 hrs.	Death in 2 min.
Apr. 14...	203	255 gm.	2 c.c.		Death in 2 min.
Apr. 14...	204	255 gm.	1 c.c.		Death in 2 min.
Apr. 14...	205	260 gm.	0.5 c.c.		Death in 5 min.
Apr. 14...	206	250 gm.	0.25 c.c.		Acute symptoms. Recovery in 15 min.
Apr. 14...	207	250 gm.	0.5 c.c.		Death in 3 min

As in the preceding experiment, here again the serum is not toxic immediately after the separation from placenta, but it becomes toxic in the absence of placenta if allowed to remain in the incubator, thus suggesting that the serum and not the placenta is the source of poison.

However, this conclusion is open to the criticism that the possibility of the serum containing minute particles of placenta in suspension after centrifugation is not definitely excluded. Such particles of placenta, although not resulting in toxicity of the serum immediately after removal from ice, may be digested later when the serum is transferred to the incubator, and thus contribute to the toxicity of the fluid. In order to show that such particles of placenta could not become the source of anaphylatoxin, the following experiment was undertaken (table V).

Pregnant human serum was placed in two centrifuge tubes, 5 c.c. in each, with about ·2 gm. of human placenta protein. In two other centrifuge tubes pregnant guinea pig serum, 5 c.c. in each, was placed, again with 2 gm. of human placenta protein. Another exactly similar series of four tubes was set up with guinea pig instead of human placenta. All the tubes were placed for sixteen hours in the ice box, at the end of which time they were centrifuged and the serum was separated. One tube of each set was transferred into the thermostat for sixteen hours, to be injected subsequently into guinea pigs; the other tube of each set was tested immediately after the separation from the substratum.

If the toxic substances in the experiments above originated from

TABLE V.

Toxicity of Sera before Incubation.

Date.	Animal No.	Weight.	Dose.	Material injected.	Results.
Apr. 17	211	265 gm.	2 c.c.	Human serum + human placenta	Death in 10 min.
Apr. 17	212	260 gm.	1.5 c.c.	Human serum + human placenta	Mild symptoms.
Apr. 17	214	250 gm.	1.5 c.c.	Human serum + guinea pig placenta	Mild symptoms.
Apr. 17	215	250 gm.	3 c.c.	Guinea pig serum + human placenta	No symptoms.
Apr. 17	216	248 gm.	4 c.c.	Guinea pig serum + guinea pig placenta	No symptoms.
Toxicity of the Same Sera after Incubation at 37° C. for Sixteen Hours.					
Apr. 18	219	255 gm.	2 c.c.	Human serum + human placenta	No symptoms.
Apr. 18	220	250 gm.	3 c.c.	Human serum + human placenta	No symptoms.
Apr. 18	221	258 gm.	4 c.c.	Human serum + guinea pig placenta	No symptoms.
Apr. 18	226	255 gm.	1 c.c.	Guinea pig serum + human placenta	Death in 5 min.
Apr. 18	227	260 gm.	0.5 c.c.	Guinea pig serum + human placenta	Death in 5 min.
Apr. 18	228	260 gm.	0.25 c.c.	Guinea pig serum + human placenta	Coughing; recovery in 15 min.
Apr. 18	229	258 gm.	0.5 c.c.	Guinea pig serum + guinea pig placenta	Death in 3 min.
Apr. 18	231	250 gm.	0.25 c.c.	Guinea pig serum + guinea pig placenta	Mild symptoms for 10 min.; recovery.
Apr. 18	233	250 gm.	0.5 c.c.	Guinea pig serum + guinea pig placenta	Death in 5 min.

the minute particles of tissue remaining in suspension after centrifuging, then the fluid injected into guinea pigs 219 or 220 and that injected into 226 or 227 should be equally toxic, since in both cases the sera were digested with the same tissue. The same should be true about the toxicity of the portions of human and guinea pig sera, respectively, both of which were digested with guinea pig placenta. As can be seen from the results, however, whereas the toxicity of guinea pig serum increased during the incubation more than 800 per cent., the human serum, on the contrary, lost its natural toxicity for guinea pigs. The results of this experiment seem to show conclusively that the toxic substances in the Abderhalden test originate from the serum.

DIGESTED SERUM IS TOXIC ONLY FOR HOMOLOGOUS ANIMALS.

Although the results of the preceding experiment show that the toxicity of the end-products of the Abderhalden reaction originates

from the serum, the fact that the pregnant human serum, having been treated in a way absolutely similar to that of a pregnant guinea pig, failed to develop toxic properties suggests further study. There seem to be two possible explanations of this difference. Either the split products of human serum are in general less toxic than those of guinea pig serum, or the products of digestion of any serum are toxic to homologous animals only. In order to determine which is the case, the following experiments were undertaken.

On Apr. 21 5 c.c. of the serum of a pregnant rabbit and guinea pig, respectively, were put in test-tubes with 2 gm. of boiled human placenta tissue in each and left on ice over night. A similar set of two tubes was made up with human placenta and male instead of pregnant serum of a rabbit and a guinea pig. Early the next morning the contents of all four tubes were centrifuged, and after separation from the sediment the sera were transferred to the thermostat for sixteen hours. At the end of this time the contents of the tubes were injected intravenously into male guinea pigs and rabbits with the results indicated in table VI.

TABLE VI.

Date	Animal.	Animal No.	Weight.	Dose.	Material injected.	Results.
Apr. 22	Rabbit	101	1,075 gm.	2 c.c.	Pregnant rabbit serum	Acute symptoms. Found dead next morning.
Apr. 22	Rabbit	102	1,090 gm.	1 c.c.	Pregnant rabbit serum	Grave symptoms. Recovered.
Apr. 22	Rabbit	103	1,015 gm.	5 c.c.	Male rabbit serum	No symptoms.
Apr. 22	Rabbit	104	1,025 gm.	3 c.c.	Pregnant guinea pig serum	No symptoms.
Apr. 22	Rabbit	105	1,025 gm.	3 c.c.	Male guinea pig serum	Slight symptoms; recovered in 15 min.
Apr. 22	Guinea pig	306	255 gm.	1.5 c.c.	Pregnant rabbit serum	Restless; recovered in 5 min.
Apr. 22	Guinea pig	307	250 gm.	1.5 c.c.	Male rabbit serum	Coughing; recovered in 30 min.
Apr. 22	Guinea pig	308	255 gm.	0.5 c.c.	Pregnant guinea pig serum	Dead in 2 min.
Apr. 22	Guinea pig	309	245 gm.	4 c.c.	Male guinea pig serum	No symptoms.

It would seem, therefore, that the products of autodigestion of human serum must be toxic for human beings if they are toxic at all. In order to determine if this was the case, serum of a pregnant woman was treated in a way exactly similar to that just described for the serum of a rabbit or guinea pig. After separation from the placenta and subsequent incubation at 37° C., it was injected at in-

tervals during the process of autodigestion in the dose of 0.05 c.c. into the skin of several members of the laboratory staff. Parallel injections were made also with 0.05 c.c. of male serum treated in a similar manner. Whereas the injections of male serum did not cause any symptoms, the pregnant serum showed unmistakable signs of toxicity. Moreover, this toxicity seemed to be increasing for a certain time with the progress of digestion, since the injection made six or twelve hours after the beginning of autodigestion did not cause any symptoms. The injection of the material digested eighteen, twenty-four, and thirty hours was followed by a strong local reaction which resulted in the first two cases in a distinct reddened area with marked sensitiveness, and in the last case in the production of a sterile pustule, which appeared about ten hours after injection and remained for over twenty-four hours (14). The reactions at the site of the inoculation with the same serum later in the process of autodigestion showed marked diminution in toxicity of the serum, and after forty-eight hours the serum lost all its toxicity.[2]

TOXICITY IS CHARACTERISTIC ONLY OF A CERTAIN STAGE OF THE AUTODIGESTION OF THE SERUM.

In the experiments reported above it was assumed on the basis of previous results that toxicity of the serum, previously treated with kaolin or placenta protein, respectively, developed during its subsequent incubation at 37° C. is due to the process of autodigestion. If this is true, the loss of toxicity of such a serum, if incubation is prolonged, may be due to the further cleavage of the split products of the serum beyond the toxic stage. That both these assumptions are correct may be seen from the following experiments (table VII).

6 c.c. of pregnant guinea pig serum were placed in four test-tubes, 1.5 c.c. in each, and left on ice for sixteen hours in contact with 0.5 gm. of boiled human

[2] Similar findings were reported by Vaughan, Cumming, and Wright (15). According to these authors the serum or extracts of organs from actively anaphylactic guinea pigs after half an hour's contact with antigen furnished (intracardial injection) poisons which were acutely fatal, while sera or extracts of organs of normal animals did not. These authors report that after longer contact these poisons were destroyed.

placenta tissue in each tube. At the end of this time the contents of all tubes were thoroughly centrifuged, the serum was separated and transferred to four tubes containing 4.5 c.c. of physiological salt solution, 4.5 c.c. of normal guinea pig serum, 4.5 c.c. of solution of serum albumin, and 4.5 c.c. of a solution of serum globulin, respectively. All the tubes were placed in the incubator and at the end of sixteen hours the contents were injected intravenously into normal guinea pigs.

TABLE VII.

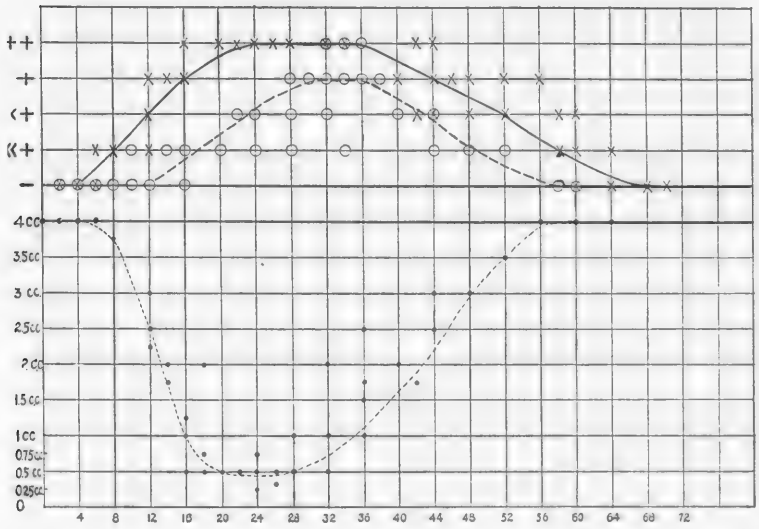
Date.	Animal No.	Weight.	Dose.	Material injected.	Results.
May 8	320	240 gm.	1 c.c.	Treated serum + salt solution	No symptoms.
May 8	321	250 gm.	1.5 c.c.	Treated serum + salt solution	Grave symptoms. Recovery.
May 8	322	250 gm.	2 c.c.	Treated serum + salt solution	Death in 2 min.
May 8	323	240 gm.	4 c.c.	Treated serum + normal serum	Very mild symptoms.
May 8	324	250 gm.	4 c.c.	Treated serum + serum albumin	Mild symptoms.
May 8	326	253 gm.	4 c.c.	Treated serum + serum globulin	Death in 2 min.
May 8	327	255 gm.	1.5 c.c.	Treated serum + serum globulin	Grave symptoms. Found dead next morning.

It is evident that the excess of normal serum or serum albumin, which, as I showed (16), stops the autodigestion of serum, as tested by the appearance of dialyzable ninhydrin reacting substances, prevents also the formation of toxic products in the serum. Serum globulin, which was shown to promote the autodigestion of serum, does not stop the formation of anaphylatoxin. That such toxic sera, during prolonged incubation, lose their toxicity due to further digestion of toxic compounds, can be seen from the following experiment.

On May 2 at 4 P.M. five pregnant guinea pigs were bled, and 40 c.c. of the serum were placed on ice in a tube with 20 gm. of boiled human placenta. At 8 A.M. on May 3, after sixteen hours, the serum was separated by centrifuging and transferred to the thermostat. In the progress of incubation samples of the serum were taken at intervals and tested simultaneously for toxicity upon normal guinea pigs as well as for dialyzable products of digestion. The latter test was done by transferring 1.5 c.c. of the serum from the incubator into a dialyzing thimble, as for the Abderhalden test (but without substratum), and placing the container with the thimble on ice. Under these circumstances, as I have shown, no further digestion takes place, and if there are any dialyzable products in the serum, they are not prevented from diffusing into the outer liquid in the container. Dialysis is allowed to proceed for twelve hours and at the end of this time the ninhydrin as well as biuret tests are made. The results of the experiment are shown in table VIII.

TABLE VIII.

Date.	Duration of incubation.	Weight.	Dose.	Results.	Biuret test.	Ninhydrin test.
May 3, 3 P.M.	6 hrs.	250 gm.	0.5 c.c.	No symptoms	−	−
May 3, 9 P.M.	12 hrs.	250 gm.	0.5 c.c.	Death in 10 min.	−	<+
May 4, 9 A.M.	24 hrs.	245 gm.	0.5 c.c.	Death in 5 min.	<+	++
May 4, 9 P.M.	36 hrs.	240 gm.	0.5 c.c.	Grave symptoms Recovery	≪+	++
May 4, 9 P.M.	36 hrs.	245 gm.	1 c.c.	Death in 10 min.	≪+	+
May 5, 9 A.M.	48 hrs.	250 gm.	1 c.c.	No symptoms	−	≪+
May 5, 9 A.M.	48 hrs.	250 gm.	2 c.c.	Mild symptoms	−	≪+
May 5, 9 A.M.	60 hrs.	255 gm.	2 c.c.	No symptoms	−	−
May 6, 9 A.M.	72 hrs.	253 gm.	2 c.c.	No symptoms	−	−



TEXT-FIG. 1. The average changes of the serum, recorded in several successive experiments. The numbers placed on abscissæ represent the intervals of time at which the examinations were made. The ordinates show the intensity of the respective reactions. The lowest dotted curve represents the toxicity developed in the serum during incubation, the ordinates expressing the volume of the minimal toxic dose, which is inversely proportional to the toxicity of serum. The two upper curves represent the intensity of the biuret (dotted line) and ninhydrin (solid line) tests and are expressed in arbitrary terms of negative, very weakly positive, weakly positive, positive, and strongly positive reactions.

The results, however, are not absolutely constant, since the rate of digestion may be influenced by the amount of antibody as well as by the antitryptic index, both of which vary in individual cases, and if both happen to exert their influence in the same direction the autodigestion may be markedly delayed or accelerated, as the case may be.

Comparison of these curves suggests definitely that the toxicity of the serum is in direct relation to its digestion, and that, if this digestion is allowed to proceed far enough, the cleavage products are broken down beyond the stage at which they possess the molecular configuration responsible both for the toxicity as well as for the typical color reactions (text-figure 1).

DISCUSSION.

Assuming that the anaphylactic syndrome, as it occurs in the body of a sensitized animal upon introduction of antigen, is due to the toxicity of the products of specific parenteral digestion of antigen, Friedberger attempted to produce the same substances *in vitro* by adding fresh complement to the antigen-antibody combination. The poisons obtained in this way, when injected in normal animals, were able to produce typical anaphylactic phenomena. Assuming that the substance obtained *in vitro* was identical with that formed *in vivo* in anaphylaxis, he called this poison anaphylatoxin. However, in the later studies of Friedberger, and especially in the work of other authors, the term anaphylatoxin was applied to any poisonous substance which was able to produce in normal animals the phenomena similar to those of anaphylactic syndrome.

Thus various authors have called anaphylatoxin the chemical poisons obtained by Vaughan from bacteria; and Nathan, Bordet, and Mutermilch called anaphylatoxins substances obtained by them from the serum by adsorption with inert substances. The attempts of these authors to explain the nature of anaphylatoxin seem to be generally inadequate, because even though, as my experiments also have confirmed, by digestion of serum with kaolin, for instance, one can produce from serum a substance which is similar in its physiological action, on the one hand, to the anaphylatoxin of Friedberger, and, on the other hand, to the chemical poison of Vaughan, its identity with one or the other is not proved by this similarity alone.

Inasmuch as my experiments show that the changes which the serum undergoes under the influence of kaolin are identical with the changes taking place in the immune serum as a result of its interaction with the antigen, they seem to throw light on the nature of anaphylatoxin. Since the process of the formation of toxic products in both cases is established as identical, the similarity of the biological properties of the respective end-products may speak for their identity. These findings, taken in connection with the results reported by me in the previous communication, suggest that the nature of anaphylatoxin is as follows: Fresh serum[3] contains normal proteolytic ferments whose digestive action *in vivo* is inhibited by the simultaneous presence of some antitryptic elements. This antitrypsin can be removed from the serum *in vitro* by two independent processes: one non-specific, a simple mechanical adsorption by means of excess of some organic as well as some inorganic substances; the other specific, an inactivation of the antitryptic properties of the serum taking place as a result of the physicochemical changes in the serum induced by the specific interaction between the antigen and the antibody of the immune serum. The removal of the inhibiting antitryptic action of the serum by either method is followed by the restitution of the activity of the normal proteolytic enzyme, which digests the globulin of the serum.[4] At a certain stage of this autodigestion the serum exhibits toxic properties which are able to cause a typical reaction (local or general) in homologous animals.

SUMMARY.

1. The union of fresh serum of pregnant or immunized animals with the corresponding boiled protein (substratum) is accompanied by the formation of poisonous substances.

[3] Heated serum is free from these ferments unless reactivated by the addition of fresh serum (complement).

[4] It is probable that such proteolytic ferments, activated (by the removal of the inhibition exerted by serum antitrypsin) through the combination of antigen and antibody *in vivo*, may attack also the sensitized circulating antigen. What is important, however, in relation to the recent theories of immunity advanced by Abderhalden, is the fact that such ferments are not specific and when placed in contact with the coagulated antigen *in vitro* are absolutely unable to digest the latter.

2. The poison originates from the serum as a result of its autodigestion, and not from the substratum.

3. The process of autodigestion may be determined by the specific or non-specific removal of the antitrypsin of the serum.

4. The poisons originating from the serum are toxic only for homologous animals.

5. The autodigestion of the serum, if allowed to proceed far enough, may go beyond the toxic stage.

6. The biological properties of these poisons indicate their close similarity to the anaphylatoxin, and suggest that the anaphylatoxin of Friedberger is a product of the autodigestion of serum, and not of the protein outside of the serum.

BIBLIOGRAPHY.

1. Friedberger, E., and Cederberg, O. A., *Centralbl. f. Bacteriol., 1te Abt., Orig.*, 1914, lxxii, 385.
2. Bronfenbrenner, J., *Proc. Soc. Exper. Biol. and Med.*, 1914, xii, 3, 4.
3. Bronfenbrenner, *Jour. Exper. Med.*, 1915, xxi, 221.
4. Doerr, R., *Wien. klin. Wchnschr.*, 1912, xxv, 331.
5. Friedberger, E., *Ztschr. f. Immunitätsforsch., Orig.*, 1909–10, iv, 636.
6. Friedberger, E., and Castelli, G., *Ztschr. f. Immunitätsforsch., Orig.*, 1910, vi, 179.
7. Friedberger, E., and Vallardi, C., *Ztschr. f. Immunitätsforsch., Orig.*, 1910, vii, 94.
8. Vaughan, V. C., and Wheeler, S. M., *Jour. Infect. Dis.*, 1907, iv, 476.
9. Ritz, H., and Sachs, H., *Berl. klin. Wchnschr.*, 1911, xlviii, 987.
10. Bordet, J., *Compt. rend. Soc. de biol.*, 1913, lxxiv, 225.
11. Nathan, E., *Ztschr. f. Immunitätsforsch., Orig.*, 1913, xvii, 478.
12. Mutermilch, S., *Ann. de l'Inst. Pasteur*, 1913, xxvii, 83.
13. Friedberger, E., *Ztschr. f. Immunitätsforsch., Orig.*, 1913, xviii, 227.
14. Bronfenbrenner, *Proc. Soc. Exper. Biol. and Med.*, 1914, xii, 48.
15. Vaughan, V. C., Cumming, J. G., and Wright, J. H., *Ztschr. f. Immunitätsforsch., Orig.*, 1911, ix, 458.
16. Bronfenbrenner, *Proc. Soc. Exper. Biol. and Med.*, 1914, xii, 6, 7.

THE WIDE-SPREAD DISTRIBUTION OF DIPHTHEROIDS AND THEIR OCCURRENCE IN VARIOUS LESIONS OF HUMAN TISSUES.*

By WILLIAM H. HARRIS, M.D., and H. WINDSOR WADE, M.D.

(*From the Laboratories of Pathology and Bacteriology of Tulane University of Louisiana, and the Charity Hospital, New Orleans.*)

Plate 37.

The term diphtheroid is here used with the meaning employed by writers in recent literature, particularly as regards the description of organisms of supposed etiological significance. It is not our intention to classify the diphtheroids as a separate species. Many writers, however, suggest an intimate relation or even identity between diphtheroids and certain streptothrices, which may exist in some instances. The term pseudodiphtheria as applied to *Bacillus xerosis* or *Bacillus hoffmanni* would seem to represent a synonym, except that by a diphtheroid an organism outside of the diphtheria group is apparently meant. By a diphtheroid is indicated, therefore, a non-acid-fast, Gram-positive bacillus resembling morphologically, at least in one or more of its forms, the bacillus of diphtheria. The diphtheroids may present either bipolar granules, barred arrangement of the chromatin, or, as is usually the case, shapes corresponding to involution types of *Bacillus diphtheriæ; i. e.,* club, retort, mandolin, or other forms of unusual morphology.

In the problems of the etiology of such diseases as leprosy, Hodgkin's disease, and general paresis, there have been described organisms which are diphtheroidal in morphology and to which causal properties have been ascribed in whole or in part. In certain instances these organisms have fallen almost into oblivion since the true causal agents have been discovered, postulated, and accepted; in others they are held partly in abeyance; while in still others certain diphtheroidal bacilli are supported, although not finally accepted.

* Received for publication, January 14, 1915.

There seems to be a tendency to view these diphtheroids, when found in the course of etiological investigation, from the standpoint of causal relation rather than from the more probable aspect of contamination. This is especially true of those organisms cultivated from tissues which are sterile as far as accidental contamination is concerned. Many strains grow but feebly, requiring special foodstuffs, while others cannot as yet be made to grow away from the original tissue, even under the most favorable conditions. It is a common practice to regard slow, delicate, or fastidious growth as indicative of pathogenicity, and to be incompatible with contaminating saprophytic properties. Therefore, it would seem that the widespread distribution of this type of organism in the human host and in nature as a whole is not fully realized. When the prevalent *Bacillus subtilis, Bacillus proteus,* staphylococcus, or other ordinary aerobic organisms are met with in bacteriologic work, they are discarded as irrelevant contaminators. However, should a diphtheroid be chanced upon, particularly if it be somewhat difficult to cultivate, one is apt to assume an etiological significance for this particular organism, and to undertake the establishment of such a property, without sufficiently considering the likelihood of its being present as an accidental or associated non-pathogen. Many diphtheroidal organisms of the acid-fast group, such as *Bacillus smegmatis,* are extremely difficult of cultivation, yet as far as is known they are non-pathogenic. The more rapidly and luxuriantly growing types of the group of diphtheroids are not easily mistaken, since they are so frequently encountered upon the skin surface, in the hair, and particularly in the protected natural cavities, the conjunctiva and genitalia. Even here, however, occasionally other types are encountered, which being apparently less saprophytic attract more attention.

In many instances organisms are obtained from fresh sterile human tissues which invite serious consideration as the causal factors of pathologic conditions on account of their source, their scarcity, their slow and often fastidious growth, and their diphtheroidal morphology. It need only be emphasized here that numerous workers have cultivated organisms from normal tissues. Wolbach and Saiki (1), after anesthetizing hea[illegible]gs and searing the abdominal

walls with hot irons and employing instruments thoroughly flamed, succeeded in cultivating from the liver of these animals in twenty-one out of twenty-three instances an anaerobic spore-bearing bacillus. Nicholls (2) and Ford (3) have also shown that bacteria can be recovered from healthy organs by cultivation. Wrosczek (4) has demonstrated that bacteria can pass from the alimentary tract by feeding cultures of non-pathogenic, pigment-producing organisms to animals and recovering these from various organs.

The various conditions in which members of this group of organisms have been isolated and considered as causal factors embrace a large number of diseases. We wish briefly to discuss the more important of these conditions.

With the exception of the study of the diphtheroids of leprosy, there has been no attempt to confirm the findings of the different workers upon the various diseases in which such organisms have been described. Our purpose is to indicate the broad latitude of this large group, the scope of which seems to have been only partially realized in the past.

The Eye.—Probably one of the first conditions to be ascribed to a diphtheroid was what is known as xerosis conjunctivæ. From this condition an organism was repeatedly cultivated which was designated *Bacillus xerosis* by Kuschbert (5) and Neisser in 1884. Its presence, however, was later shown to be incidental and not connected with the production of the condition. Under the name of *Bacillus xerosis,* as pointed out by Park and Williams (6), observers have described organisms of widely varying characteristics, which serves to illustrate the general confusion concerning the majority of these organisms of the diphtheroid group as a whole.

Brain and Cerebrospinal Fluids.—The repeated recovery of an organism of a diphtheroid nature from the brains of general paretics led Robertson (7), Hoag (8), Robertson (9), McRae and Jeffry, and others to regard it as probable that this organism was the pathogenic factor, although no experiment furnished sufficient weight for the final acceptance of this hypothesis. Orr, Rows, and Robertson (10) considered that they had substantiated *Bacillus paralyticus* of Robertson by the experimental histopathological lesions produced in the rabbit. Gay and Southard (11), in the examination of cerebro-

spinal fluids removed post mortem from the brains of fourteen cases of general paresis, failed to obtain a diphtheroid in any instance. The demonstration of *Treponema pallidum* in the brains of such conditions by Noguchi and Moore (12), Noguchi (13), and by Nichols and Hough (14) has banished all thought of this factor. Here again we have a striking example of the mistaken impressions received concerning the part of the diphtheroids isolated from tissues. In this connection it should be noted that in the routine study of cerebrospinal fluids, diphtheroidal organisms are sometimes encountered, the significance of which seems to vary in different cases. To illustrate this fact we shall cite a few typical instances. One fluid which when freshly drawn was reported negative for bacteria was found to contain after two days' standing numerous thin, beaded, sharp-ended, Gram-positive diphtheroids, easily grown in pure culture. A second case, that of a four day infant, showed at autopsy a general purulent meningitis from extension of a *Staphylococcus aureus* infection of an ulcerated spina bifida sac. Twenty-four hour cultures made from the spinal meninges showed numerous staphylococci with a few colonies of a Gram-positive diphtheroid. In both cases the organisms were evidently contaminators. It is more difficult, however, to account for the organism found in a third case. This was an adult male with high temperature and opisthotonus. On lumbar puncture forty cubic centimeters of a slightly turbid fluid were removed, which upon immediate centrifugalization showed many pus cells and numerous large, irregular, Gram-positive bacilli, varying from solid coccoid forms to those simulating the large, barred, club forms of *Bacillus xerosis*. These were found to be still numerous in a specimen taken twenty-four hours later, but subsequently they diminished in number until they disappeared, and the case recovered about a week after the first puncture. Upon none of the ordinary media, aerobically or anaerobically, could more than a slight, temporary multiplication be obtained.

Blood Cultures.—In the course of routine blood culture work, diphtheroids occasionally have grown out on the medium employed either together with organisms such as *Bacillus typhosus* and *Streptococcus pyogenes* or in pure culture. Their occurrence in conjunction with known pathogens, together with the fact that refinement

of technique diminishes the frequency of their occurrence, stamps them as frank contaminators. Their isolation, therefore, in blood culture from diseases of unknown etiology, such as typhus fever (15), etc., must be regarded with due conservatism. The recovery of non-pathogenic, non-toxin-producing strains of supposed *Bacillus diphtheriæ* in cases considered to be diphtheria septicemia, in certain instances may be thus explained. That there may occasionally occur more or less generalized invasion of diphtheroids, however, is held probable. An instance suggesting this was encountered in a case of systemic blastomycosis which came to autopsy. In the purulent material from abscesses in the neck and pancreas and from cavities in the lungs which contained numerous yeast cells there were demonstrated in smears many Gram-positive diphtheroids. Cultures were obtained from these lesions which proved to be identical.

An interesting case that might be mentioned here was one in which pus was obtained upon aspiration of the chest, that showed in stained smears very numerous thin, non-acid-fast diphtheroids, easily grown in pure culture. The case subsequently recovered and was lost sight of.

Lymphoid Tissues.—During the past summer lymph nodes, usually inguinal or femoral, have been aspirated in approximately thirty cases presenting themselves at the Charity Hospital, in order to determine the possibility of *Bacillus pestis* infection. In several of these cases a few diphtheroids have been found in the smears obtained by puncture which with a simple stain were bipolar. As has been pointed out by one of us (16), it is important to bear this fact in mind in the study of simple stained smear preparations for the diagnosis of early plague infection. From three of these lymph node aspirations cultures of these confounding organisms have been recovered. It is worthy of note that their presence seems much more frequent in chronic hyperplasias than in acute infections of lymph nodes.

The lymphadenopathy which constitutes the lesion known as Hodgkin's disease has but recently been ascribed to a diphtheroidal type of organism. From the lesions Fraenkel and Much (17), de Negri and Mieremet (18), Bunting and Yates (19), Billings and Rosenow (20), Lanford (21), and others have reported the recovery

of diphtheroids of pigment- and non-pigment-producing varieties. Lanford reports the recovery of two strains of these bacilli from the spleen of splenic anemia. Bunting, Yates, and Kristjanson (22) have recently reported the cultivation of their *Bacillus hodgkini* from the spleen of so called splenic anemia. They argue from these findings a relation between this condition and Hodgkin's disease. We have cultivated diphtheroidal organisms from tuberculous glands, lymphosarcoma, and Hodgkin's disease, but have not attempted to carry out a comparative study of our cultures and those isolated from Hodgkin's disease by other workers. Strains of diphtheroids resembling those recovered from lesions of Hodgkin's disease have been cultivated by various workers from splenic anemia, lymphosarcoma, tuberculous adenitis, and other lesions. While lesions have been produced locally by the injection of these organisms, no general picture of the disease has resulted. Still it must be remembered that generalized lesions have been produced with known saprophytes intravenously injected in large amounts and at sufficiently frequent intervals. Again, in studies bearing upon the causation of hepatic cirrhosis de Vieche (23) by introducing certain bacteria into the intestinal tract, and Weaver (24) by subcutaneous injection of bacilli of the colon group obtained definite proliferation of the interstitial connective tissue of the liver. In this connection Hektoen's work (25) is of special interest, as he produced similar lesions with a pseudodiphtheria or diphtheroidal organism. Furthermore, we can here refer to the acid-fast group which forms a more striking analogue for confusion in the identification of the Hansen bacillus culture. Duval and Harris (26) and Machow (27) have produced definite generalized nodular lesions in rabbits and white rats with intravenous injection of such saprophytes as the bacillus of timothy hay, *Bacillus smegmatis,* and the dung bacillus of Moeller.

Tumors.—In order to ascertain whether diphtheroids were present in tumors, bits of tissue from six specimens, usually warm from the operating room, were planted on various media. These specimens included excised nodules of two cases of lymphosarcoma, two leiomyomata, one from the uterus and the other from the back, a fibroma removed from the pectoral muscle, and a necrotic area of an hepatic carcinoma obtained post mortem. In all cases the surface

of the tissue was thoroughly seared and the material for cultivation removed from the deeper levels. After periods ranging from three days to two weeks colonies of diphtheroids of different types appeared on or about certain of the planted bits from all these except the uterine leiomyoma. In three cases diphtheroids alone were cultivated; while from the tumor of the back the staphylococcus, and from the liver the colon bacillus were also obtained. In several instances the formation of the colony within the tissue fragment, its enlargement, and final eruption upon the surface were watched, thus eliminating the possibility of contamination through faulty technique.

In extension of this work several cultures of more or less rapidly growing diphtheroids were obtained from other tissues removed at surgical operation and planted upon nutrient media. One of these organisms is of particular interest since it belongs to the acid-fast group. From an unopened appendix, supposedly sterile, a bit of the meso-appendix was removed and planted upon blood serum. After two weeks there appeared upon the tissue itself a cadmium yellow colony. Smear preparations when stained showed a typical Gram-positive diphtheroid, which when stained by Gabbett's method proved to be strongly acid-fast. Subplants grew well upon the ordinary laboratory media and liquefied completely blood serum and gelatin. The culture has not been definitely identified, but is apparently one of the many saprophytic acid-fast strains. This isolation presents an instance of the liability of occasional contamination with a strongly acid-resisting saprophyte in cultivation experiments.

Leprosy.—Kedrowski (28), Bayon (29), Williams (30), Wolbach and Honeij (31), and others have isolated and cultivated from the leprosy lesion diphtheroids to which they have assigned definite causal properties, each regarding his culture as a stage in the life cycle of the Hansen bacillus. Kedrowski (32), in particular, reasons a mutation of the non-acid-fast diphtheroid cultivated by him from the tissue of lepra nodules, because upon injection into animals, he recovered only an acid-fast growth. Campana (33) and Babes (34), who separated non-acid-fast diphtheroids and streptothricial forms supposedly in pure cultures from leprous tissues, consider that Kedrowski was working originally with a contaminated culture. Throughout this experimental work upon the cultivation of *Bacillus*

leprœ, conducted by Duval and his assistants (35, 36), we have from time to time isolated similar non-acid-fast diphtheroids which when injected into animals have never shown evidence of having acquired acid-fast properties. These strains of diphtheroids have been maintained unchanged in culture for several years.

This confusion of acid-fast and non-acid-fast organisms recovered from the lesions of leprosy has a parallel in the following study of tuberculous tissues. For example, we have repeatedly recovered cultures of non-acid-fast diphtheroids from tissues, which by sections and smears have proved to be tuberculous. Several tissue bits were removed from a tuberculous axillary node. One of these was macerated and injected into a guinea pig that died of tuberculosis in five weeks. The remaining tissue fragments were planted in six tubes of various culture media and after incubating for one week, five of these showed staphylococci, either alone or mixed with diphtheroidal bacilli. In one tube the tissue appeared to have remained sterile. Smears from this, however, showed numerous small groups of diphtheroids, which were apparently in pure culture and subsequently became a comparatively free grower. The original tissue culture was later inoculated into a guinea pig, and after six weeks the animal showed signs of tuberculosis. From the lesions *Bacillus tuberculosis* was cultivated, but no non-acid-fast diphtheroid. In such an instance the contaminating diphtheroid and the specific acid-fast bacillus were known entities, and one cannot reason that the non-acid-fast diphtheroid is part of the life cycle of *Bacillus tuberculosis*. That Kedrowski (32) was handling a mixture of an acid-fast bacillus and a non-acid-fast diphtheroid, injection of which mixture into an animal occasioned the disappearance of the non-acid-fast diphtheroid and the recovery of the acid-fast organism, is suggested by this experiment.

For the purpose of ascertaining the liability of skin contamination in the leper, we obtained material from the Louisiana Leper Home. Nodules of tissue in the tubercular form were removed from areas which were previously sterilized with soap and water and tincture of iodin. Portions were planted upon agar, Loeffler's blood serum, and numerous special media. Surface cultures were made from the lesions of trophic types of the disease, as represented by cases with

contractures and atrophies and the partly or completely cicatrized areas of previous lesions. Similar cultures were made from macular lesions. As controls and to ascertain the prevalence of this type of organism upon the skin of lepers, similar cultures were made from uninvolved skin areas of eleven cases of various types of leprosy. Growths appeared in from one to ten days, and in many instances showed staphylococci and other contaminating organisms. In some the diphtheroids were so overgrown that they could not be recovered. The results are shown in table I.

TABLE I.

Leprosy.

No. and types of cases.	Media.	Results.
15 tissue bits, from 5 nodules excised from 4 patients with tubercular type	Tryptophane agar, tryptophane blood agar, tryptophane ascitic agar, and ordinary blood serum and nutrient agar	7 growths of diphtheroids from 4 cases. 3 heavy growers, one being dirty yellow, another dull red, and the third white. 4 slow growers, growth simulating that of *Streptococcus pyogenes*. All were non-acid-resisting.
5 plants from scrapings over nodules of 5 other cases with tubercular type	Blood serum and nutrient agar	2 growths of free growing diphtheroids. Non-acid-resisting.
10 plants from scrapings of macular lesions of 10 such cases	Blood serum and nutrient agar	No diphtheroids found.
12 plants from scrapings of lesions of 12 cases with trophic type	Blood serum and nutrient agar	9 growths of diphtheroids, 8 free growing, of which 4 were cream color and 4 white, 1 slow grower, streptococcus-like. 2 of these cultures showed streptothrices.
2 plants from conjunctival sac and 1 from a comedo	Blood serum and nutrient agar	All showed diphtheroids, those from the eye corresponding to *B. xerosis*.
11 plants from normal skin areas of 11 patients with various types	Blood serum and nutrient agar	6 growths of diphtheroids. Three were creamy, two lemon yellow, and one slow growing and streptococcus-like. 2 of these cultures showed streptothrices.

Summary.—From 45 different lepers presenting various forms of the disease, 27 growths of chromogenic, non-chromogenic, free growing, and slow growing diphtheroids were obtained. Six of these were from normal skin areas. It is noteworthy that none of these

organisms were acid-resisting when the ordinary standard methods of decolorization were employed.

All the various morphological characteristics of *Bacillus diphtheriæ* were represented in these cultures. Many of them were shown to be weakly acid-resisting, if the mineral acids used to decolorize were as dilute as 2.0 per cent.

These results indicate the prevalence of bacilli of the diphtheroid type and the possibility of encountering them where the cultivation of *Bacillus leprœ* is attempted.

Skin.—To determine the presence of diphtheroids in miscellaneous skin lesions, cases of pellagra, acne, marked comedones, etc., were selected. The skin areas were carefully cleansed with 95 per cent. alcohol, and the pus, parts of the scales, and the comedones were cultured on ordinary laboratory foodstuffs. Forty such plants were made, and while a large number contained staphylococci, twenty-five showed definite diphtheroidal bacilli, many of which were isolated in pure culture. Twenty-three were of a rapidly growing character, and two grew slowly and scantily. The majority of these diphtheroids presented a moist cream colored growth; one was salmon in color, one yellow, and some were quite white. All stained by Gram's method, and the salmon colored chromogenic strain was acid-resisting. This particular organism was recovered from a moist eczematous lesion of the neck. In two of the twenty-five tubes containing diphtheroids, unidentified streptothrices were found.

Various workers such as Castellani (37), Chalmers and O'Farrall (38), Chalmers and Stirling (39), and Johns (40) have cultivated from the hairs of trichonocardiasis a bacillus which they regard as an integral unit of the life cycle of the *Nocardia tenuis;* they also procured colonies of cocci. The bacillus from description and plates is of a distinctly diphtheroidal character. While, of course, we recognize that this type of organism might form a phase of higher plant life, still because of the presence of known irrelevant organisms in some of their cultures and in the light of the extensive presence of diphtheroids on the skin, the question of contamination is here again to be considered. We may mention particularly a case regarded by the dermatologist as probable sporotrichosis, which recovered rapidly with antisyphilitic treatment. Three nodular lesions were present,

one on the dorsum of the wrist and two on the forearm. After careful sterilization the skin was incised and several subcutaneous tissue bits were removed. These were planted in many tubes of various media, and after incubation for six days colonies appeared on the tissue bits in two of the tubes. Both were chromogenic. One was yellow, grew well in subplant, and presented chiefly short chubby bacilli, while the other was of a light yellow color and grew very feebly, showing marked pleomorphism.

The great liability of skin contamination by diphtheroids is again demonstrated by their cultivation from the various cutaneous disturbances selected for cultural study.

Air.—A series of eighteen plates measuring fourteen centimeters in diameter was exposed to the air for varying periods of time. Nine of these contained blood agar, and six contained blood serum. As an occasional liquefier destroys a plate of blood serum, blood agar was found to be preferable. Thirty minutes' exposure yielded a colony seeding which could be advantageously picked after the necessary incubation. Three different plates were exposed on different days so that the daily air currents would present different atmospheres for examination; likewise different rooms were chosen to form a varied environment of search. The plates were incubated for forty-eight hours, with results which are shown in table II.

TABLE II.

Aerobic Organisms.

Plates.	Time.	Results.
4 plates, 14 cm. (2 of blood agar and 2 of blood serum)	15 min.	3 colonies of diphtheroids.
10 plates, 14 cm. (8 of blood agar and 2 of blood serum)	30 min.	29 colonies of diphtheroids. 7 colonies distinctly acid-fast.
4 plates, 14 cm. (2 of blood agar and 2 of blood serum)	45 min.	6 colonies of diphtheroids.

Summary.—18 plates showed 45 colonies of diphtheroids. Of these, 7 were of an acid-fast nature. They were mostly chromogenic and varied as follows: 17 salmon, 7 chocolate brown (probably in part due to the pigment of the blood agar), 6 lemon yellow, 5 pink, 4 white, 3 distinctly red, and 3 were colorless and grew slowly.

The colonies varied in size from 0.5 mm. to 4 mm., averaging about 2.5 mm. They were round and were sharply defined in contour. All were moist with the

exception of the three small, colorless colonies which grew slowly in transplants and assumed the appearance of an influenza culture.

The results demonstrate that diphtheroidal bacilli are readily obtained from the atmosphere, and show the possibility of contamination through this means.

Aerobic diphtheroids have been recovered by McNaught (41) from water. We have also isolated them from urine and feces.

DISCUSSION AND SUMMARY.

It is evident from this work and from the work of others that organisms having diphtheroidal morphology are wide-spread in their distribution in the human body. They are readily isolated from miscellaneous skin lesions, probably thriving better in pathological soils as saprophytes than in normal skin, although they can be obtained quite easily from the latter. Various strains can also be cultivated from the air, at least in this environment.

Aside from the air and superficial parts of the human body, diphtheroids have frequently been isolated from the deeper regions. Hoag, working on general paresis and certain conditions of the lung, cultivated 199 diphtheroids, the majority of which were from the lungs and respiratory tract, also in some instances from the liver and spleen. He considered 146 of these isolations to be identical and classified them as organism x. Robertson, McRae, and Jeffry likewise in cases of dementia paralytica isolated what they considered an identical strain which was closely related to the Klebs-Loeffler bacillus, from the inflamed ileum, stomach, tonsils or pharynx, bronchi, lung, and brain. They differentiated their organism from *Bacillus hoffmanni* and *Bacillus xerosis.*

The fact that these organisms have a somewhat unusual morphology, resembling as they do an organism of known pathogenicity, namely, *Bacillus diphtheriæ,* should form no basis for assuming that they, too, are apt to possess these qualities. When we consider the number of simple bacillary contaminators analogous to such organisms as *Bacillus anthracis, Bacillus typhosus,* etc., we can readily see how frequently this occurs.

The majority of the diphtheroids isolated by us stain by Gram's

method; nearly all fail to resist the standard acid-fast methods, although use of the very low strengths of mineral acids allows the fixed stain to remain in whole or in part. There is consequently nothing unique in their tinctorial properties.

These bacillary strains differ considerably in their powers of cultivation. While the majority grow readily upon the ordinary laboratory foodstuffs, others require special products for their growth and still others cannot be grown even where special effort has been made. The fact that many strains grow slowly no doubt enhances their consideration as disease producers, as it seems to argue their difficult adaptation from their preëxisting site to an artificial environment. This reasoning does not apply when we consider the numerous causal agents that can so readily be cultivated directly from the diseased tissue, and again the fact that many saprophytes in their natural habitat are incapable of growth *in vitro* (figure 1).

The most striking of the biological features of these cultures is their wide range of pigment production. While many are white or creamy white, the majority range from a canary yellow to a distinct crimson, and one of the cultures recovered from a leper was a very deep dusky red or garnet. Numerous combined shades of yellow and pink, including such colors as salmon and orange, were present. Some cultures were distinctly pink, others varied in depth of color to a crimson. While many cultures of identical colors were obtained, the majority of strains showed some slight variance of shade.

While, of course, the inoculation of diphtheroidal cultures produces varying degrees of localized inflammation if employed in sufficient dosage, probably for the two-fold reason of acting as a foreign body and liberating proteolytic substances, still the diphtheroids isolated in the past have never been able to reproduce the clinical picture of the various diseases from which they were cultivated. The apparent reproduction of lesions in general paresis, in hepatic cirrhosis, and in leprosy does not form an ultimate postulate for the acceptance of organisms as specific etiological entities. This is evidenced by the fact that certain known saprophytic organisms when used as controls are capable of producing the same results.

It seems clear that organisms as broadly distributed as the diph-

theroids should be suspected as contaminators when met in experiments bearing upon etiology. They should be viewed primarily in the same light as the staphylococcus and the various known contaminating bacteria. It is apparent that this type of organism has been regarded too much from the standpoint of pathogenicity. It is, of course, probable that among this large group there may be a few pathogenic types.

CONCLUSIONS.

1. Diphtheroids are widely distributed in nature. They are present in the air, on the body surface, and at times through contamination or are indigenous in the deeper tissues.

2. Diphtheroids constitute a broader field of saprophytism than is generally appreciated. While some strains may represent pathogens, their aggregate is patently not of the disease-producing variety.

3. Diphtheroids can be cultivated from various pathological tissues to which they bear no etiological relation, such as lesions of tuberculosis, leprosy, blastomycosis, tertiary syphilis, and tumors of various types.

We wish to extend our thanks to Dr. C. W. Duval for suggestions, and to Dr. A. Mattes and Miss Alice B. Moerner for assistance in certain features of this work. We wish also to thank Dr. Ralph Hopkins, Visiting Physician in charge of the Louisiana Leper Home, who made it possible to obtain certain material.

BIBLIOGRAPHY.

1. Wolbach, S. B., and Saiki, T., *Jour. Med. Research,* 1909, xxi, 267.
2. Nicholls, A. G., *Canadian Jour. Med. and Surg.,* 1899, vi, 405.
3. Ford, W. W., *Tr. Assn. Am. Phys.,* 1900, xv, 389.
4. Wrosczek, cited by Adami, J. G., The Principles of Pathology, Philadelphia and New York, 1910, i, 321.
5. Kuschbert, *Deutsch. med. Wchnschr.,* 1884, x, 321, 341.
6. Park, W. H., and Williams, A. W., Pathogenic Micro-Organisms Including Bacteria and Protozoa; A Practical Manual for Students, Physicians and Health Officers, New York and Philadelphia, 1910, 206.
7. Robertson, W. F., *Brit. Med. Jour.,* 1905, ii, 1427.
8. Hoag, L., *Boston Med. and Surg. Jour.,* 1907, clvii, 10.
9. Robertson, W. F., *Brit. Med. Jour.,* 1903, ii, 1065.
10. Orr, D., and Rows, R. G., *Proc. Path. Soc. of Great Britain and Ireland, Jour. Path. and Bacteriol.,* 1910, xiv, 134. Robertson, W. F., *Jour. Path. and Bacteriol.,* 1910, xiv, 134, 145.

11. Gay, F. P., and Southard, E. E., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1910, lv, 117.
12. Noguchi, H., and Moore, J. W., *Jour. Exper. Med.*, 1913, xvii, 232.
13. Noguchi, H., *Jour. Am. Med. Assn.*, 1913, lxi, 85.
14. Nichols, H. J., and Hough, W. H., *Jour. Am. Med. Assn.*, 1913, lxi, 120.
15. Plotz, H., *Jour. Am. Med. Assn.*, 1914, lxii, 1556.
16. Wade, H. W., and Staring, H. L., *Am. Jour. Trop. Dis.*, 1914, ii, 260.
17. Fraenkel, E., and Much, H., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1910, lxvii, 159.
18. de Negri, E., and Mieremet, C. W. G., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1913, lxviii, 292.
19. Bunting, C. H., and Yates, J. L., *Arch. Int. Med.*, 1913, xii, 236; *Jour. Am. Med. Assn.*, 1913, lxi, 1803.
20. Billings, F., and Rosenow, E. C., *Jour. Am. Med. Assn.*, 1913, lxi, 2122.
21. Lanford, J. A., *Am. Jour. Trop. Dis.*, 1914, ii, 191.
22. Yates, J. L., Bunting, C. H., and Kristjanson, H. T., *Jour. Am. Med. Assn.*, 1914, lxiii, 2225.
23. de Vieche, B., *Sperimentale*, 1899, liii, 199.
24. Weaver, G. H., *Tr. Chicago Path. Soc.*, 1900, iii, 228.
25. Hektoen, L., *Jour. Path. and Bacteriol.*, 1901, vii, 214.
26. Duval, C. W., and Harris, W. H., *Jour. Med. Research*, 1913–14, xxviii, 165.
27. Machow, D., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1913, lxvii, 434.
28. Kedrowski, W. J., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1901, xxxvii, 52.
29. Bayon, H., *Brit. Med. Jour.*, 1911, ii, 1269.
30. Williams, T. S. B., *Brit. Med. Jour.*, 1911, ii, 1582.
31. Wolbach, S. B., and Honeij, J. A., *Jour. Med. Research*, 1914, xxx, 1.
32. Kedrowski, W. J., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1910, lxvi, 1.
33. Campana, R., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1910, lxvii, 361.
34. Babes, V., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1911, lix, 493.
35. Duval, C. W., *Transactions of the XVIIth International Congress of Medicine, Section IV, Bacteriology and Immunity*, London, 1913, pt. ii, 103.
36. Duval and Harris, *loc. cit.*, p. 171.
37. Castellani, A., *Brit. Jour. Dermat.*, 1911, xxiii, 341; *Tr. Roy. Soc. Med.*, 1912, vi, 23.
38. Chalmers, A. J., and O'Farrall, W. R., *Ann. Trop. Med. and Parasit.*, 1913, vii, 525.
39. Chalmers, A. J., and Stirling, A. D., *Ann. Trop. Med. and Parasit.*, 1913, vii, 541.
40. Johns, F. M., *Am. Jour. Trop. Dis.*, 1913–14, i, 782.
41. McNaught, J. G., *Jour. Roy. Army Med. Corps*, 1909, xii, 655.

EXPLANATION OF PLATE 37.

The illustration affords some idea of the growth and appearance of representative isolations in the chrome series.

Cultures D 17, D 29, and D 32 are from tumors, and D 21 is from an appendix removed at operation. Cultures D 74, D 75, D 76, D 79, D 81, D 85, and D 88 are chromes recovered from air. D 58 is from the feces of a case dead of *B.*

aërogenes capsulatus infection. D 52 was obtained from a blood culture. D 49 is one of the chromogenic strains cultivated from acne.

D 17, D 21, D 29, and D 52 present irregular, granular, wrinkled growths, somewhat dry in appearance. Culture D 21 imbeds itself slightly in blood serum, possessing to a minor extent a liquefying property.

D 49, D 58, D 79, D 81, and D 85 show a more or less smooth surface and are moist.

D 32, D 74, D 75, D 76, and D 88 grow very heavily, appearing quite thick; the surfaces are smooth and glistening, and the growths are extremely moist.

The chromes produced by these cultures are as follows: 17, a cream yellow; 21, pink; 29, deep orange; 32, red; 49, pink, but darker than 21, and its growth is smooth; 52, cream white or ivory; 58, a very dark pink approaching red; 74, salmon; 75, cadmium; 76, orange; 79, canary yellow; 81, cream; 85, yellow; 88, a pinkish salmon.

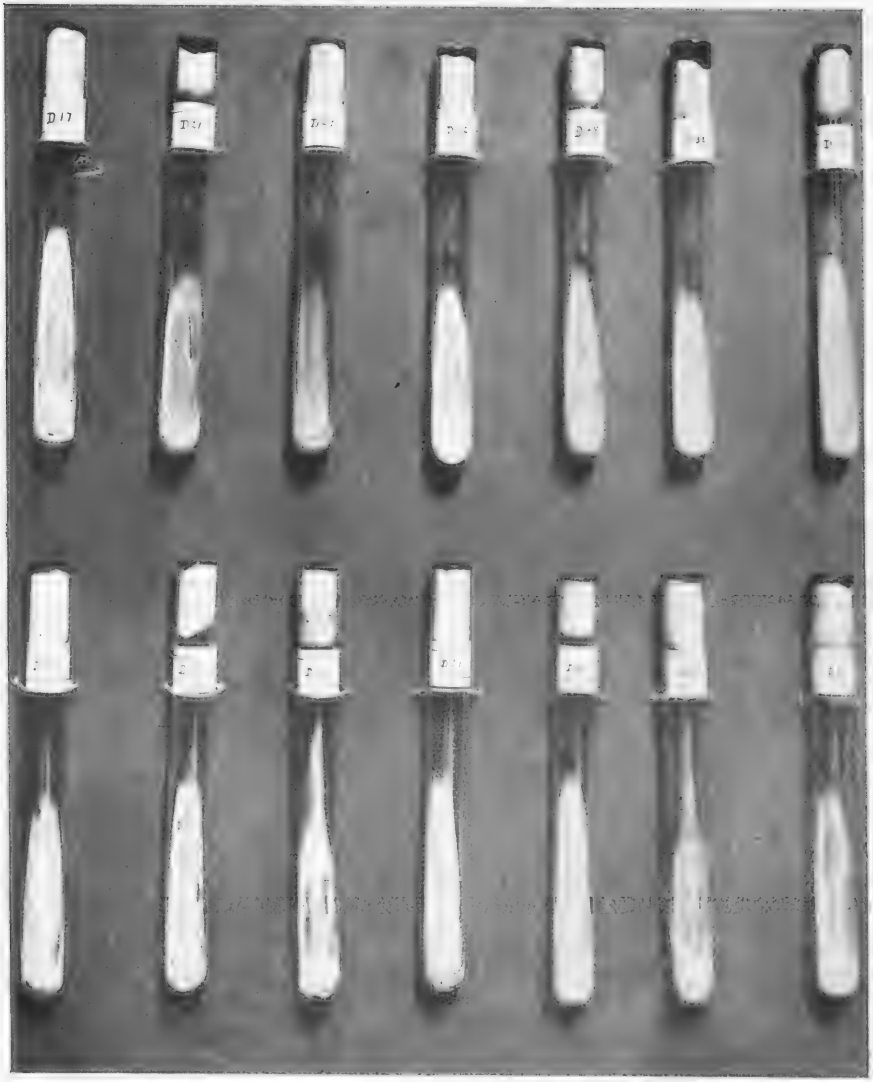


FIG. 1.

(Harris and Wade: Distribution of Diphtheroids.)

DIFFUSION AND SURVIVAL OF THE POLIOMYELITIC VIRUS.*

By SIMON FLEXNER, M.D., and HAROLD L. AMOSS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

In earlier publications[1] we have presented data bearing on the view that the minute microörganism cultivated from poliomyelitic tissues and described by Flexner and Noguchi,[2] constitutes the cause of epidemic poliomyelitis. In this paper we propose to deal with the power of diffusion and the period of survival of the virus of poliomyelitis contained in the central nervous organs, since these factors affect the evidence submitted concerning the specific activities of the microörganism.

We have alluded in a previous paper to the factor of the survival of the virus at thermostatic temperature,[3] but this has never been accurately determined and could only be inferred from experiments made to determine other qualities of the virus. The question of the power of diffusion of the virus from a fragment of infected nervous tissue into the surrounding medium has never been directly considered.

The capacity for diffusion and survival of the virus bears directly upon the evidences that have been brought forward to support the specific nature of the microörganism under consideration. We now know that certain cultural strains after a number of removals from the nervous tissues from which they were derived, and great dilution, may still set up poliomyelitis in monkeys upon inoculation. Although the experiments demonstrating this fact are convincing, it is nevertheless desirable to ascertain by direct experiment what the capacity for diffusion and survival of the ordinary virus of

* Received for publication, March 1, 1915.

[1] Flexner, S., and Noguchi, H., *Jour. Exper. Med.*, 1913, xviii, 461. Flexner, S., Noguchi, H., and Amoss, H. L., *ibid.*, 1915, xxi, 91.

[2] Flexner and Noguchi, *loc. cit.*

[3] Flexner, Noguchi, and Amoss, *loc. cit.*

poliomyelitis is, in order to exclude the remote possibility of the persistence within the cultures of an active agent of invisible character.

EXPERIMENTAL.

The method employed to study the diffusion and survival of the virus is simple. Fragments of brain tissue containing the virus are placed in tubes of ascitic fluid and kidney fragment overlaid with paraffin oil, the medium employed for actual cultivation tests. The tubes are not enclosed in anaerobic jars, and are kept at 37° C. From time to time a tube is removed from the thermostat, and a quantity of the ascitic fluid in proximity to the brain tissue is pipetted off, after which the brain fragment is removed. Both fluid and fragment are used for inoculating monkeys by intracerebral injection. Since the tubes have not been enclosed in an atmosphere of hydrogen, multiplication of the minute organism will probably not take place. Whether or not it does take place can be determined by suitable tests.

It is essential to employ a strain of the virus of high virulence. This was done in the experiments by selecting a monkey from which to take the brain fragments, which had succumbed to an inoculation of a highly active sample of the ordinary virus of poliomyelitis. The virus is capable of producing paralysis when injected intracerebrally in quantities of 0.1 to 0.3 of a cubic centimeter of a Berkefeld filtrate of a 5 per cent. emulsion of the spinal cord. Before the brain tissue submitted to incubation is employed for inoculation, an emulsion is prepared containing 0.2 of a gram of the tissue which inoculated intracerebrally into a control animal causes paralysis in a period varying from five to eight days. Subsequently it is necessary merely to remove the inoculated tissue at stated intervals from the thermostat and to inoculate the corresponding quantity of 0.2 of a gram of brain fragment, in order to determine the period during which the virus survives in an active state under the conditions of the experiment.

The tests on the diffusion of the virus were conducted with quantities of 0.2 of a cubic centimeter of the ascitic fluid adjacent to the brain fragment which had been pipetted off at intervals after

incubation. The quantity of 0.2 of a cubic centimeter was chosen because it represents the average quantity of the fluid medium which is transferred from the original to subsequent tubes of ascitic fluid medium in the course of the cultivation of the minute microorganism from infected brain tissue. Should the virus diffuse freely from the brain tissue into the surrounding medium, the ascitic fluid should then become infectious. No experiments have ever been conducted to ascertain whether diffusion takes place at all, or the extent to which it does occur. Should the diffusion be so slight that 0.2 of a cubic centimeter of the ascitic fluid contains less than a minimal dose capable of producing poliomyelitis in the monkey, the transfer of this quantity of the fluid medium from one tube to another could not result in the carrying over of an effective amount of the virus.

INOCULATION OF BRAIN TISSUE.

Experiment 1.—Control. Jan. 7. Injected intracerebrally into a *Macacus rhesus* an emulsion containing 0.2 gm. of brain removed from a recently paralyzed monkey. Jan. 10. Excitable. Jan. 11, A.M. Tremor, ataxia. P.M. Arms and legs weak; later prostrate. Jan. 12. Died. Typical poliomyelitis.

Experiment 2.—Brain tissue incubated for 10 days. Jan. 18. Injected intracerebrally into a *Macacus rhesus* 0.2 gm. of brain tissue, in emulsion, derived from the same animal as in experiment 1, which had been incubated in ascitic fluid kidney medium for 10 days. Jan. 31, A.M. Arms paralyzed, paralysis of right side of face. P.M. Prostrate. Feb. 1. Etherized. Typical poliomyelitis.

Experiment 3.—Brain tissue incubated for 20 days. Jan. 28. Same procedure as in experiment 2. Feb. 4. Excitable, ataxic, left arm paralyzed. Feb. 6. Prostrate. Feb. 8. Died. Typical poliomyelitis.

Experiment 4.—Brain tissue incubated for 30 days. Feb. 9. Same procedure as in experiment 2. No effects produced.

Experiment 5.—Brain tissue incubated for 40 days. Mar. 1. Same procedure as in experiment 2. No effects produced.

INOCULATION OF ASCITIC FLUID.

Experiment 6.—Ascitic fluid incubated for 10 days. Jan. 18. 0.2 c.c. of ascitic fluid adjacent to brain tissue employed in experiment 2, removed by pipette after 10 days' incubation, inoculated intracerebrally into a *Macacus rhesus*. No effects produced.

Experiment 7.—Ascitic fluid incubated for 20 days. Feb. 1. Same procedure as in experiment 1. No effects produced.

Experiment 8.—Ascitic fluid incubated for 30 days. Feb. 9. Same procedure as in experiment 1. No effects produced.

DISCUSSION.

The experiments described indicate that, with a highly active virus, 0.2 of a gram of the brain tissue taken from a recently paralyzed monkey will cause certain paralysis in a *Macacus rhesus* when injected by the intracerebral route. When, however, specimens are taken from the same portions of the brain from which the test fragment was removed and incubated in the ascitic fluid kidney medium, they still cause paralysis when similarly inoculated into a *Macacus rhesus* on the tenth or the twentieth day of incubation, but not on the thirtieth or the fortieth day. But the ascitic fluid adjacent to the brain fragments fails to set up paralysis in a *Macacus rhesus* when injected intracerebrally in quantities of 0.2 of a cubic centimeter after an incubation of ten, twenty, or thirty days.

The meaning of the experiments is clear. At the temperature of the thermostat, the ordinary virus of poliomyelitis survives in the autolyzing brain fragment containing it for a period of about twenty days, while it seems incapable of diffusing from the fragment into the surrounding medium in considerable quantity during the period of its active survival in the tissues.

The period of survival of the virus at 37° C. indicated by the experiments is almost exactly paralleled by the tests made by Levaditi[4] with fragments of infected intervertebral ganglia which he submitted to cultivation *in vitro*. Levaditi observed that the longest period at which the virus could still be detected in an effective state was twenty-one days, which period agrees exactly with our observations. Levaditi also noted that only the intervertebral ganglionic tissue, and not the surrounding plasma, was infectious. From this he concluded that the virus which he believed to have increased, multiplied only in association or symbiosis with nerve cells; but the observation is readily explained on the supposition that failure to detect the virus in the plasma was due to lack of its diffusion from the ganglionic tissue into the surrounding medium.

Hence the experiments performed for the express purpose of determining the limits of survival and degree of diffusion of the

[4] Levaditi, C., *Compt. rend. Soc. de biol.*, 1913, lxxiv, 1179; 1913, lxxv, 202.

virus at 37° C. have yielded results which coincide almost exactly with the results attributed by Levaditi to multiplication of the virus *in vitro* in association with proliferating tissue cells. It is highly probable that in Levaditi's experiments no multiplication of the virus whatever took place; and, moreover, that the discrepancy in respect to infectiousness observed between the ganglionic tissue and the plasmatic medium is explained, not by the proliferation of the virus within the former, but by the failure of diffusion into the latter.

Our experimental results are supported by observations recorded by Flexner and Noguchi,[5] which indicated that when no demonstrable growth of the microörganism has taken place in the ascitic fluid medium, the original tube, as well as the fluid in the early transfers, is wholly without power to incite poliomyelitis upon inoculation into monkeys.

The cultures, it will be recalled, are developed in the ascitic fluid kidney medium from fragments of infected brain tissue. From the original cultures obtained, subcultures are made by transferring after an inoculation of one or two weeks quantities of the ascitic fluid varying in amount from 0.2 to 0.5 of a cubic centimeter. The possibility of transferring in this way from one tube to another a certain quantity of the original virus of poliomyelitis contained within the brain fragment has always been considered. On the other hand, it has been pointed out that with each successive transfer, so rapid a dilution of any original virus present would doubtless take place that the fluid would soon lose infectious power from this source.[6] It now, however, appears that the quantity of virus which diffuses from the brain fragment into the ascitic fluid is so small that it may be disregarded from the outset.

Similar considerations relate to the survival of the virus in the brain tissue itself employed for the culture medium. Since the period of this survival is only about twenty days, cultures which have been incubated for weeks or months, and which are still active, cannot owe their infective power to the original virus, but must owe it to the multiplied microörganism. Moreover, when the

[5] Flexner and Noguchi, *loc. cit.*

[6] Flexner, Noguchi, and Amoss, *loc. cit.*

additional fact is taken into account that the successfully inoculated cultures were often many generations removed from the brain fragment from which they were derived, and in one instance had remained in the thermostat for a period exceeding one year, further consideration need hardly be devoted to the possibility that the experimental poliomyelitic infection is attributable in part to the coöperation of unchanged virus and culture of the minute microorganism.

CONCLUSIONS.

The ordinary virus of poliomyelitis present in aseptically removed brain tissue of paralyzed monkeys survives in an ascitic fluid kidney medium at the temperature of 37° C. for a period of at least twenty, but not of thirty days.

Under the conditions of moderate anaerobiosis, the minute microorganism cultivated from poliomyelitic tissues tends not to develop in cultures from the brain tissue; hence its presence does not complicate the survival test.

The diffusion of the ordinary poliomyelitic virus from a noncomminuted fragment of brain tissue into a surrounding medium of ascitic fluid is so slight as not to be detectable by inoculation experiments conducted with usual quantities of the fluid.

The specific effects of the microörganism cultivated from poliomyelitic tissues are not caused by an admixture in the cultures of the ordinary virus of poliomyelitis; hence they must be caused by the pathogenic action of the microörganism itself.

The minute microörganism is therefore to be regarded as the specific microbic cause of epidemic poliomyelitis.

THE RAPID PRODUCTION OF ANTIDYSENTERIC SERUM.*

By SIMON FLEXNER, M.D., and HAROLD L. AMOSS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

A side issue of the European war has been the unexpected and great demand for certain therapeutic sera. Among those for which a sudden demand has arisen is the antidysenteric serum, of which in the recent past large quantities have not been needed. It is doubtful whether the serum was being produced in notable quantities in any of the countries involved in the war, so that when dysentery appeared among the troops engaged in the western campaign, supplies of the serum were sought outside.

Among the early activities of The Rockefeller Institute was included a systematic investigation,[1] in the eastern part of the United States, of the bacteriology of acute dysentery and related diseases of the intestine. An important incident of this investigation was the preparation and testing upon human subjects of the antidysenteric serum. Doubtless, it was the knowledge of these studies possessed abroad that led to the requests made upon The Rockefeller Institute for supplies of the serum. This demand, which was met inadequately by supplies then available, led us to undertake the improvement of the method of preparing the serum, with the particular object in view of reducing the period required for the immunization of horses.

The outlook for success in this endeavor was hopeful, since the experience of the past few years had shown that certain antisera could be prepared in small animals—the rabbit especially—in far shorter periods of time than had been supposed. We owe especially to the

* Received for publication, March 1, 1915.

[1] Flexner, S., and Holt, L. E., Bacteriological and Clinical Studies of the Diarrhœal Diseases of Infancy. Report of the Rockefeller Institute for Medical Research, New York, 1904.

studies of Fornet and Müller,[2] Bonhoff and Tsuzuki,[3] and Gay[4] and his pupils the discovery of the fact that the reinjection of antigenic substances at brief intervals leads to an intensification of the antibody formation. In this manner and through three successive daily injections of protein, bacterial or body cells, strong precipitating, agglutinating, and cytolytic substances have been evoked. We have applied the principles of this method to the immunization of a horse with several types of the dysentery bacilli, with the result to be described.

VARIETIES OF DYSENTERY BACILLI.

Two distinct groups of bacilli capable of causing dysentery are now distinguished. As far as is known, the members of the group bring about similar or indistinguishable intestinal lesions and clinical symptoms.[5] As regards ordinary morphological and cultural characters, the groups are also indistinguishable; while they are readily distinguished by their power to ferment certain carbohydrates and alcohols (especially mannite), and by the important fact that one group yields in the cultures a soluble toxin and the other does not.

The original of the dysentery bacilli is designated the Shiga[6] bacillus, from the name of the Japanese bacteriologist who first isolated and studied the dysenteric bacillus. It happens that the Shiga bacillus possesses limited fermentative power and produces a soluble toxin. Dysenteric bacilli identical with the Shiga bacillus have been obtained everywhere in the Eastern, Western, and Southern Hemispheres in which they have been sought by bacteriologists. Hence the Shiga bacilli constitute the original group of dysenteric

[2] Fornet, W., and Müller, M., *Ztschr. f. biol. Techn. u. Method.*, 1908, i, 201.

[3] Bonhoff, H., and Tsuzuki, M., *Ztschr. f. Immunitätsforsch., Orig.*, 1909–10, iv, 180. Tsuzuki, M., *ibid.*, p. 194.

[4] Gay, F. P., and Fitzgerald, J. G., *University of California Publications in Pathology*, 1911–14, ii, 77. Gay, F. P., *Ergebn. d. Immunitätsforsch., exper. Therap., Bakteriol. u. Hyg.*, 1914, i, 231. Locke, E., *University of California Publications in Pathology*, 1911–14, ii, 91.

[5] The question whether the Shiga bacillus is less often, and the Flexner group of bacilli more often present in sporadic cases of dysentery is still an open one; but members of both groups may cause epidemics.

[6] Shiga, K., *Centralbl. f. Bakteriol., 1te Abt.*, 1898, xxiii, 599.

bacilli. They also compose an homogeneous group, since all the Shiga bacilli so far studied possess identical properties.

This is not true of the second group, which includes several quite well defined members. The first representative of this group was isolated by Flexner[7] from cases of acute dysentery occurring in the Philippine Islands. Hence in contradistinction to the Shiga group, it has become customary to speak of the other as the Flexner group. But, as already indicated, the latter is heterogeneous and contains two or more other varieties of closely related but yet distinguishable bacilli, sometimes designated as Y[8] and Strong.[9] None of the varieties in this group yields a soluble toxin; and while all show wider fermentation powers than the Shiga bacillus, yet the several members differ in that respect among themselves. Very recently Sonne[10] has described still another variant belonging in this group, which he cultivated from several cases of acute dysentery occurring in Copenhagen.

But the most important distinguishing feature exhibited by the dysentery bacilli relates to their antigenic or immunity properties. By employing especially the agglutination test, and to a less extent the test of protection, the Shiga and Flexner groups are more sharply, and the several members of the latter group less sharply separated from one another.

As regards the practical question of the specific therapy of dysentery, the most important consideration is that of protection. It is manifestly impossible to undertake to discriminate in many instances among cases of dysentery occurring sporadically in endemics or epidemics, the particular dysenteric bacillus causing the infection before applying the serum treatment. Two ways of proceeding are, therefore, indicated: first, to determine whether an antidysenteric serum prepared with the bacilli of one group protects against the bacilli of the other group; and second, to ascertain whether an effective polyvalent antidysenteric serum suitable for use irrespective of the type of infecting bacillus can be prepared.

[7] Flexner, S., *Philadelphia Med. Jour.*, 1900, vi, 414.

[8] Hiss, P. H., Jr., and Russell, F. F., *Med. News*, 1903, lxxxii, 289.

[9] Strong, R. P., and Musgrave, W. E., Report of the Etiology of the Dysenteries of Manila. Report of Surgeon General of the Army to the Secretary of War for 1900, Washington, 1900.

[10] Sonne, C., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1915, lxxv, 408.

Indeed, it has long been known that the dysenteric bacilli possess both common and specific antigenic properties; a certain amount of cross-agglutination and protection exists which is more pronounced between the related members of the Flexner group than between the more widely separated Flexner and Shiga groups. Again, it has been found practicable to produce in a given horse by suitable methods of immunization antibodies for the two groups of bacilli, and in quantities which promise to suffice for practical therapeutic purposes. This fact is not only important as regards the treatment of cases of bacillary dysentery infected with a single strain,—the rule among cases ordinarily arising,—but is significant also in respect to the rarer instances of mixed strain infection, in which more than one member of the Flexner group, or in which the Shiga bacillus and a member of the Flexner group coöperate in causing the lesions and other effects of the disease.

RAPID PREPARATION OF POLYVALENT ANTIDYSENTERIC SERUM IN HORSES.

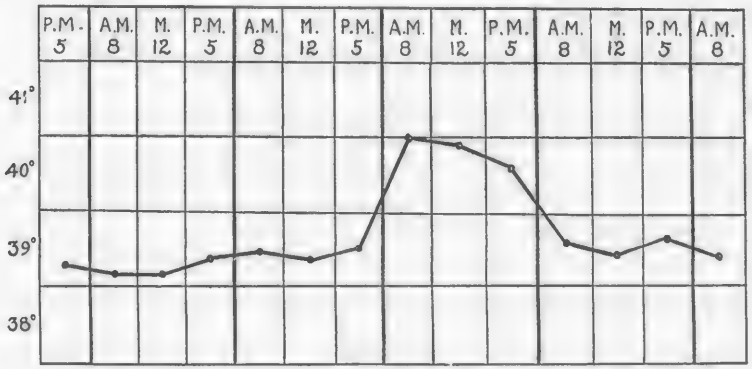
Hitherto horses have been injected with cultures or soluble products of the dysenteric bacilli over long periods of time. The injections have been made chiefly into the subcutaneous tissues, rarely into the veins. The severe reactions which intravenous injections often cause limited that method of immunization. The subcutaneous injections, on the other hand, are more readily carried out, but at the stage of the immunization process when large quantities of culture—or, in the case of the Shiga bacillus, soluble toxin—must be injected, large infiltrations may arise which sometimes ulcerate and depress the physical condition of the animals. The process of immunization is then halted until the ulcer heals and the general condition improves, entailing a loss of time; but in some instances a state of cachexia arises, leading to loss by death. By employing the subcutaneous method of immunization, an active antiserum can be prepared in from about nine to twelve months.

The process can be abbreviated by employing the method of successive intravenous inoculations over brief periods of time, with intervening periods of rest. The principles of the method are those developed by Fornet and Müller, Bonhoff and Tsuzuki, and Gay and

his pupils. By injecting successively the several varieties of dysenteric bacilli, a serum possessing high agglutinative and protective value has been secured in a period measured by weeks rather than by months.

Cultures.—The cultures employed were made upon agar-agar slant surfaces in tubes 15 by 160 mm. in size. The cultures were employed after twenty-four hours of incubation at 37° C., and the growth in each tube was suspended in 2 c.c. of 0.8 per cent. salt solution, from which measured quantities were taken for intravenous injection. The several members of the Flexner group and several strains of the Shiga bacillus were employed for injection.

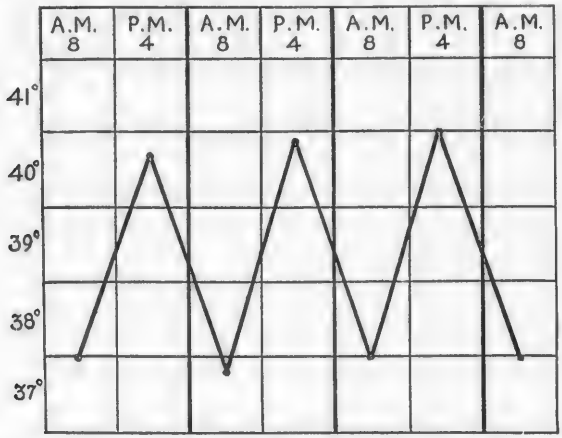
Injection.—Blood having been drawn in order to secure a sample for the control tests of agglutination and protection, the preliminary injection of cultures was made with 1 c.c. of the suspension of Flexner bacilli which had been heated to 60° C. for thirty minutes. The febrile reaction was slight. Twenty-four hours later 5 c.c. of the suspension were injected, which was followed by a sharp reaction (text-figure 1). The third injection was deferred forty-eight



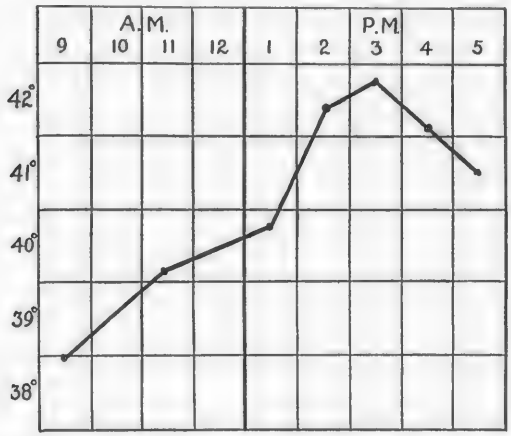
TEXT-FIG. 1. Febrile reaction to Flexner bacilli killed by heat.

hours, until the temperature again reached normal, when 5 c.c. of the suspension were again injected. After an interval of seven days, living cultures were substituted for the heated ones. The febrile reactions following the injection of four loops of the suspension on the first, ten loops on the second, and twenty loops on the third day were negligible. At the third inoculation suspensions of living Shiga bacilli were injected. The dose was four loops on the first, ten on the second, and thirty on the third day. From this time on, Flexner and Shiga bacilli were inoculated alternately on three successive days with intervening rest intervals of seven days, the doses being chosen so as to produce a sharp febrile reaction which subsided in twenty-four hours. By adjusting the

doses with greater care, the febrile curve was rendered typical (text-figure 2). As a rule, the dose of a culture which was injected on the third day of the last period was employed for injection on the first day of the next period. The



TEXT-FIG. 2. Typical febrile reaction from three injections on successive days.



TEXT-FIG. 3. Typical febrile rise and fall following each single injection.

increase in dosage was made by quite regular increments; namely, by fractions of + 1/8, + 1/5, etc., of the quantity injected on the preceding day. It was ascertained that twenty loops of the culture weighed about 0.15 gm. When

the quantity of culture injected once exceeded twenty loops, the measurement of the suspension was made in graduated pipettes. From time to time blood was drawn for titration; it was taken just preceding a new series of inoculations, or, in other words, seven days after the last inoculation of the preceding series.

Temperature.—The temperatures were taken about one hour before each injection. It was noted that the maximal febrile point was reached about six hours after inoculation, after which the descent began, the normal being reached usually at about the end of the twenty-four hour period, which was the result strived for. The inoculations were made at about 9 A.M., and the temperatures were taken the fifth, sixth, and seventh hours afterwards, or until the figure indicated that the maximum had been reached and decline had begun (text-figure 3).

IMMUNITY VALUE OF THE ANTISERUM.

The degree of immunity produced by the several successive inoculations was ascertained by titrating the antiserum at intervals against the Shiga and Flexner groups of bacilli in respect to (a) the agglutination, and (b) the protective value.

Agglutination.—The titer of the horse serum before inoculation was begun for the several varieties of bacilli employed for injection is shown in table I. The Flexner group, as was previously known, is more readily agglutinated than the four strains of Shiga bacilli employed, although the Flexner D proved inagglutinable in the unheated serum.

TABLE I.

Agglutination Titer of Normal Horse Serum.

Variety of dysentery bacilli.		Limits of agglutination.	
		Unheated.	Heated to 56° C.
Flexner group	Flexner	1:60	1:120
	Strong	1:60	1:120
	Y	1:60	1:120
	Flexner D	0	0
	Shiga 293	1:30	1: 20 (partial).
	Shiga G	1:20 (partial)	1: 20 (partial).
	Shiga-Kruse	1:30	1: 20
	Shiga K	1:20 (partial)	1: 20

The rapidity in the rise of the agglutination titer after each successive series of three injections of the bacilli is shown in table II. The values given are for complete agglutination in serum not inactivated by heat. Heating the serum to 56° C. reduced the agglutina-

TABLE II.

Periodic Test of Agglutination Value for Flexner and Shiga Groups of Bacilli.

Variety of bacilli.	1st period.[11]	2d period.	3d period.[13]	4th period.	5th period.	6th period.	7th period.	8th period.	9th period.	10th period.
Flexner.....	1:500	1:800	1:1400	1:1500	1:2000	1:2000	1:3000	1:4000	1:4000	1:4000
Strong.....	1:400	1:800	1:800	1:1400	1:1800	1:1800	1:3000	1:4000	1:4000	1:4000
Y.....	1:300	1:800	1:800	1:1500	1:1800	1:2200	1:2300	1:3000	1:5000	1:5000
Flexner D[12]..	0	0	0	0	0	0	0	0	0	0
Shiga 293....	—	—	1:80	1:90	1:500	1:500	1:1000	1:1800	1:3000	1:4000
Shiga G.....	—	—	1:70	1:100	1:150	1:200	1:600	1:700	1:1200	1:1500
Shiga-Kruse..	—	—	1:100	1:90	1:200	1:200	1:600	1:800	1:1000	1:1200
Shiga K.....	—	—	1:80	1:150	1:150	1:200	1:600	1:700	1:1000	1:1200

tion value by half or more. The values, moreover, are given for each bleeding period, which correspond with each successive set of inoculations of the bacilli. Hence the effect of each series of three injections can be followed. In the first period, killed bacilli of the Flexner group were injected; subsequently only living bacilli were employed. The effect of the first set of injections of the Shiga bacilli is seen in the third period. The rise in agglutinins for the Flexner group is more pronounced than for the Shiga group of bacilli. The inequalities as measured by the several varieties in each group are small, except in the culture denominated Flexner D, which appears to be inagglutinable.

Protection.—The therapeutic value of the serum was tested experimentally in guinea pigs and rabbits. The anti-infectious power was determined in the former and the antitoxic power for Shiga toxin in the latter animals.

The method employed was to mix suspensions of the living cultures with dilution of the serum or of Shiga toxin with serum, and to incubate the mixtures for one hour at 37° C., after which they were injected. The injections were made into the peritoneal cavity of guinea pigs weighing about 225 gm., and into the ear vein in rabbits weighing 1,250 to 1,500 gm.

The bacilli were cultivated on slant agar surfaces and the growths were of twenty-four hours' duration. The suspensions were made in sterile normal salt solution. The Shiga toxin consisted of a growth three days old of the Shiga bacillus in sugar-free bouillon containing calcium carbonate. The bacilli were killed with ether. The ether having been removed, the bouillon culture was passed through hardened filter paper and preserved in the refrigerator. Ether was found superior to phenol for killing the bacilli. It does not injure the toxin.

[11] After inoculation of cultures killed by heat.

[12] Probably inagglutinable.

[13] The first inoculation of Shiga bacilli given in previous period.

The protective value of the antiserum was compared with that of normal serum withdrawn from the horse before inoculation was begun. The effects of the normal serum are shown in table III.

TABLE III.

Anti-Infectious and Antitoxic Value of Normal Horse Serum.

Flexner bacillus.			Shiga bacillus.			Shiga toxin.		
M.l.d.	Serum.	Result.	M.l.d.	Serum.	Result.	M.l.d.	Serum.	Result.
2	0.5 c.c.	Recovered	2	0.5 c.c.	Recovered	4	0.5 c.c.	Died.[14]
2	0.5 c.c.	Recovered	2	0.4 c.c.	Died	4	0.3 c.c.	Died.
2	0.2 c.c.	Died	2	0.3 c.c.	Died	4	0.2 c.c.	Died.
2	0.2 c.c.	Died	2	0.2 c.c.	Died	4	0.1 c.c.	Died.
2	0.1 c.c.	Died	2	0.1 c.c.	Died			
2	0.1 c.c.	Died						
2	0.05 c.c.	Died						
2	0.05 c.c.	Died						
2	0.01 c.c.	Died						
2	0.01 c.c.	Died						
2	0.008 c.c.	Died						
2	0.008 c.c.	Died						

The anti-infectious power of the normal serum was determined with two minimal lethal doses of the cultures, and the antitoxic power with four minimal lethal doses of the toxin. In the former instance, the total volume of each of the mixtures was two, in the latter one cubic centimeter.

Normal horse serum exerts slight protection only against the infectious or toxic effects of the dysentery bacilli. The limit of activity for two minimal lethal doses of the living cultures and for four minimal lethal doses of Shiga toxin is about 0.5 of a cubic centimeter of the normal serum.

With this value are to be compared the values of the serum after ten weeks' immunization which are shown in table IV. For the corresponding Shiga and Flexner bacilli it is 0.008 and for the Shiga toxin 0.003 of a cubic centimeter.

The protective and agglutination values resemble each other in appearing early and rising rapidly in the course of immunization. In view of this rapid development the serum should be suitable for therapeutic employment in man at the end of eight or ten weeks' immunization. Doubtless the continuation of the inoculations

[14] Death delayed somewhat.

TABLE IV.

Anti-Infectious and Antitoxic Value of Immune Horse Serum.

Flexner bacillus.[15]			Shiga bacillus.			Shiga toxin.		
M.l.d.	Serum.	Result.	M.l.d.	Serum.	Result.	M.l.d.	Serum.	Result.
2	0.1 c.c.	Recovered	2	0.1 c.c.	Recovered	4	0.5 c.c.	Recovered.
2	0.05 c.c.	Recovered	2	0.05 c.c.	Recovered	4	0.4 c.c.	Recovered.
2	0.02 c.c.	Recovered	2	0.01 c.c.	Recovered	4	0.2 c.c.	Recovered.
2	0.01 c.c.	Recovered	2	0.008 c.c.	Recovered	4	0.1 c.c.	Recovered.
2	0.008 c.c.	Recovered	2	0.003 c.c.	Died	4	0.008 c.c.	Recovered.
2	0.008 c.c.	Died	2	0.001 c.c.	Died	4	0.005 c.c.	Recovered.
2	0.005 c.c.	Died	2	0.0005 c.c.	Died	4	0.003 c.c.	Recovered.
2	0.003 c.c.	Died				4	0.002 c.c.	Died.
2	0.002 c.c.	Died				4	0.001 c.c.	Died.

would result in still further accumulation of specific antibodies. But it seems safe to begin regular bleeding of the horses treated in the manner described at the tenth week of the immunization process and to carry out regular bleeding with intervening inoculation from that period on.

SUMMARY.

Antidysenteric serum can be safely prepared in the horse by the method of three successive intravenous injections of living cultures or toxin with intervening rest periods of seven days.

When this method of immunization is employed, the specific antibodies responsible for agglutination and protection appear early and rise rapidly.

By inoculating alternately living dysentery bacilli belonging to the Shiga and Flexner groups a polyvalent serum of high titer may be prepared.

A polyvalent serum so produced should be suitable for the therapeutic treatment of acute bacillary dysentery, irrespective of the particular strain or strains of the dysentery bacillus causing the infection.

An effective antidysentery serum suitable for therapeutic employment in man can be prepared in the horse in about ten weeks.

[15] The experiment was done in duplicate.